(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 September 2001 (13.09.2001)

PCT

(10) International Publication Number
WO 01/66149 A2

(51) International Patent Classification[7]: A61K 48/00

(21) International Application Number: PCT/US01/06953

(22) International Filing Date: 2 March 2001 (02.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/187,236 3 March 2000 (03.03.2000) US
60/261,751 16 January 2001 (16.01.2001) US

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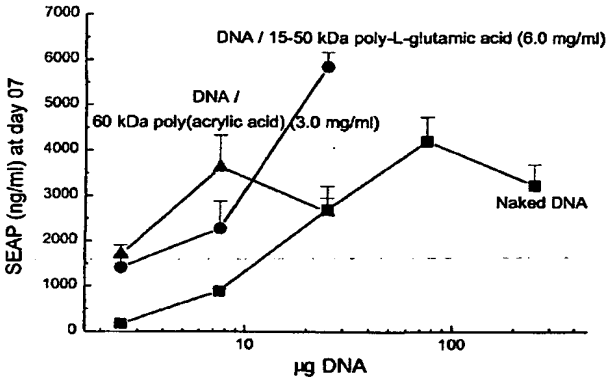
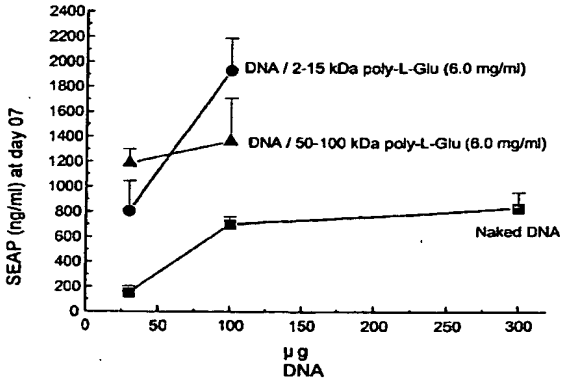
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,

[Continued on next page]

(54) Title: NUCLEIC ACID FORMULATIONS FOR GENE DELIVERY AND METHODS OF USE





(57) Abstract: A nucleic acid formulation for use in gene delivery comprising a nucleic acid and an anionic polymer is disclosed. Examples of the anionic polymer includes anionic amino acid polymer or poly-amino acid (such as poly-L-glutamic acid, poly-D-glutamic acid, poly-L-aspartic acid, poly-D-aspartic acid), poly-acrylic acid, polynucleotides, poly galacturonic acid, and poly vinyl sulfate.

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NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

DESCRIPTION

Nucleic Acid Formulations for Gene Delivery And Methods of Use

Introduction

This invention relates to novel compositions and methods for the introduction of a nucleic acid molecule into a cell, including by a pulse voltage delivery method, for the expression of a protein, peptide, antisense RNA, ribozyme, or polypeptide. Priority is claimed from United States Provisional Application Serial No. 60/187,236 filed March 3, 2000 and United States Provisional Application Serial No. 60/261,751 filed January 16, 2001, which are hereby incorporated by reference as if fully set forth herein.

Background of the Invention

The following information is presented solely to assist the understanding of the reader. None of the information is admitted to describe prior art to the claims of the present invention.

Gene therapy is a major area of research in drug development. Gene therapy has been considered a desirable mechanism to correct genetically determined diseases resulting from the failure to produce certain proteins and acquired diseases such as autoimmunity and cancer. One example of a class of genetically determined diseases that are considered amenable to gene therapy is hemophilia. Hemophilia B, for example, is a bleeding disorder that results from the absence functional blood clotting Factor IX ("F.IX"). The disease

state is classified as severe, moderate or mild, depending on the level of functional F.IX. (Lusher, J.M. (1999) *Thromb Haemost* 82:572-5751). Approximately 5,200 males are afflicted with the disease in the U.S. with approximately 45% of these cases being of the severe type. In severe cases of hemophilia B (<1% of normal F.IX levels) there are frequent bleeding events that can be life threatening and often produce debilitating destruction of the patient's joints. The current therapy for hemophilia B is the administration of F.IX protein in response to bleeding events only. The use of either blood derived or recombinant F.IX has shown that tremendous clinical and quality of life benefits can be achieved by converting the most severe hemophilia B cases into the moderate or mild range. In some countries F.IX protein is given prophylactically in the most severe cases, despite the fact that these treatments are extremely expensive (Ljung, R.C. (1999) *Thromb Haemost* 82:525-530). The prophylactic use of F.IX is not frequent in the U.S.

Gene therapy could provide a new prophylactic approach for the treatment of diseases such as hemophilia B. A technological barrier to commercialization of gene therapy, however, is the need for practical, effective and safe gene delivery methods. In animal models of hemophilia, viral-based vectors have been used successfully to administer the human F.IX gene either to liver or muscle. (Kay, M.A., et al. (1993) *Science* 262:117-119; Herzog, R.W., et al. (1999) *Nat Med* :56-63; Snyder, R.O., et al. (1999) *Nat Med* 5:64-70; Chao, H., et al. (1999) *Gene Ther* 6:1695-1704; Lozier, J.N., et al. (1999) *Blood* 94:3968-3975; Kaufman, R.J. (1999) *Hum Gene Ther* 10:2091-2107). In some cases, these approaches have led to long-term (> 2 years) expression of therapeutic levels of F.IX in a

canine model of hemophilia B (Herzog, R.W., et al. (1999) *Nat Med* 5:56-63). However, the limitations of viral-based approaches have been extensively reported. For instance, re-administration is not possible with these vectors because of the humoral immune response generated against the viral proteins. In addition to manufacturing challenges to obtain adequate reproducible vector supply, there are also significant safety concerns associated with viral vectors, particularly for those targeting the liver for gene expression. Not withstanding the problems associated with viral gene therapy, viruses have been considered by many to be more efficient than non-viral delivery vehicles.

A problem of non-viral gene therapy is to achieve the delivery and expression of sufficient nucleic acid to result in a tangible, physiologically relevant expression. Although DNA plasmids in isotonic saline (so-called 'naked' DNA) were shown several years ago to transfect a variety of cells *in vivo*, the lack of stability of such unprotected plasmids to enzymatic degradation is associated with irreproducibility in uptake leading to highly variable expression and biological responses in animal models. The very low bioavailability of 'naked' plasmid in most tissues also requires high doses of plasmids to be administered to generate a pharmacological response.

The field of non-viral gene delivery has therefore been directed to the development of more efficient synthetic delivery systems able to increase the efficiency of plasmid delivery, confer prolonged expression and provide for storage stable formulations as is expected of other pharmaceutical formulations.

To overcome the problem of degradation of nucleic acids, typically plasmid DNA ("pDNA"), and enhance the efficiency of gene transfection, cationic condensing agents (such as polybrene, dendrimers, chitosan, lipids, and peptides) have been developed to protect pDNA by condensing it through electrostatic interaction. (A. P. Rolland, From genes to gene medicines: recent advances in nonviral gene delivery, review in *Therapeutic drug carrier systems*, 15(2):143-198 (1998).) However, the use of condensed plasmid particles for transfection of a large number of muscle cells *in vivo* has not been successful as compared directly to "naked" DNA. Wolff, J. A., et al., *J. Cell Sci.*, 103, 1249, 1992. In particular, due to the physiology of the muscle, the use of rigid condensed particles containing plasmid for efficient transfection of a larger number of muscle cells has not been successful to date because cationic lipid and polylysine plasmid complexes do not cross the external lamina to gain access to the caveolae and T tubules. *Id.*

Additional strategies that include the modulation of the plasmid surface charge and hydrophobicity by interaction with protective, interactive non-condensing systems (e.g., PINC™ polymers) have shown advantages over the use of 'naked' DNA for direct administration to solid tissues. [WO9621470, US Patent No. 6,040,295, incorporated herein by reference.]

Biodegradable microspheres have also been used in gene delivery that encapsulate the nucleic acid. For example, WO0078357,Chen, W. et al, disclosed matrices, films, gels and hydrogels which include hyaluronic acid (HA) derivatized with a dihydrazide and crosslinked to a nucleic acid forming slow release microspheres. WO9524929, Boekelheide, K. et al.,

disclosed encapsulation of genes in a matrix preferably in the form of a microparticle such as a microsphere, microcapsule, a film, an implant, or a coating on a device such as a stent. US6048551, Beer, S. et al. disclosed a controlled release gene delivery system utilizing poly (lactide-co-glycolide) (PLGA), hydroxypropylmethyl cellulose phthalate, cellulose acetate phthalate, and the Ludragit R, L, and E series of polymers and copolymer microspheres to encapsulate the gene vector. Luo D et al. Pharm Res 1999 Aug;16(8):1300-8, reported the characterization of systems for controlled delivery of DNA from implantable polymer matrices (EVAc: poly (ethylene-co-vinyl acetate)) and injectable microspheres (PLGA and PLA: poly (D, L-lactide-co-glycolide) copolymer and poly (L-lactide), respectively). Despite their promise, microspheres can pose manufacturing difficulties and can adversely constrain the release of DNA *in vivo*, particularly in muscle tissue.

Thus, despite these recent advances, there remains a need for additional and improved formulated nucleic acid compositions and methods of administering the same for gene therapy.

Summary of the Invention

An alternative approach to the use of viral vectors is the use of non-viral plasmid-based gene therapy. The present invention discloses novel compositions and methods for enhancing the administration of nucleic acids and uptake thereof by an organism. In one embodiment, the formulation utilizes anionic polymers such as poly-amino acids, polynucleotides, or poly-acrylic acids that are able to enhance the transfection of nucleic acids to muscle tissues with and

without electroporation. In one embodiment of the invention, the poly-amino acid is poly-glutamic acid and salt thereof. The poly-glutamic acid formuation has been shown in the present invention to be particularly useful in increasing electroporation assisted transfection *in vivo*.

The compositions of the present invention that are used to administer nucleic acid, preferably by pulse voltage delivery, allows for treatment of diseases, vaccination, and treatment of muscle disorders and serum protein deficiencies.

Another aspect of the present invention provides a method for treating a mammalian condition or disease. The method involves the step of administering to a mammal suffering from the condition or disease a therapeutically effective amount of a composition of the invention. In one embodiment of the invention, the disease is characterized by insufficient levels of active Factor IX. Delivery of a nucleic acid encoding Factor IX formulated in poly-glutamate and delivered in conjunction with electroporation according to the present invention is able to provide nanogram levels of Factor IX in the peripheral blood of large animals.

In one embodiment of the invention, the disease is characterized by insufficient levels of red blood cells resulting in anemia. Delivery of a nucleic acid encoding erythropoietin ("EPO") formulated in poly-L-glutamate and delivered in conjunction with electroporation according to the present invention is able to provide sufficient levels of EPO to result in a maximal hematocrit level.

In one embodiement of the invention, the disease is characterized by disregulation of the immune system. Delivery of a nucleic acid encoding a cytokine, such as in one example,

human interferon alpha 2b ("hINFα"), formulated in poly-L-glutamine and delivered in conjunction with electroporation according to the present invention is able to provide nanogram levels of hINFα in the peripheral circulation.

In yet another aspect, the invention also features a method for delivering a nucleic acid molecule to a mammal, more preferably a human, by utilizing a non-condensing anionic polyamino acid formulation. The method involves the step of providing a composition of the invention to the cells of the organism by use of a device configured and arranged to cause pulse voltage delivery of the composition.

In preferred embodiments the device for delivering is an electroporation device that delivers the composition of the invention to the cell by pulse voltage and/or delivers the composition of the invention by subjecting the cells to an electric field.

The present invention also features a kit. The kit includes a container for providing a composition of the invention and either (*i*) a pulse voltage device for delivering the composition of the invention to cells of an organism, wherein the pulse voltage device is capable of being combined with the container, or (*ii*) instructions explaining how to deliver the composition of the invention with the pulse voltage device. Thus the "container" can include instructions furnished to allow one of ordinary skill in the art to make compositions of the invention. The instructions will furnish steps to make the compounds used for formulating nucleic acid molecules. Additionally, the instructions will include methods for testing compositions of the invention that entail establishing if the nucleic acid molecules are damaged upon injection after

electroporation. The kit may also include notification of an FDA approved use and instructions.

A method for making a kit of the invention is also provided. The method involves the steps of combining a container for providing a composition of the invention with either (*i*) a pulse voltage device for delivering the composition of the invention to the cells of an organism, wherein the pulse voltage device is capable of being combined with the container, or (*ii*) instructions explaining how to deliver the composition of the invention with the pulse voltage device.

The invention also provides a method of treating a mammal suffering from cancer or an infectious disease. The method involves the step of providing a composition of the invention to cells of the mammal by use of a device configured and arranged to provide pulse voltage delivery of a composition of the invention to cells of the mammal, wherein the molecule encodes a cancer antigen or an antigen for the infectious disease.

As noted above, the compositions of the present invention that are used to administer nucleic acid, preferably by pulse voltage delivery, include a compound that protects the nucleic acid and/or prolongs the localized bioavailability of the nucleic acid and/or enhances expression when administered to an organism *in vivo*, or *in vitro* in cell culture.

As the compositions are useful for delivery of a nucleic acid molecule to cells *in vivo*, in a related aspect the invention provides a composition at an *in vivo* site of administration. In particular, this includes compositions for

delivering a nucleic acid molecule at an *in vivo* site in a mammal.

The summary of the invention described above is not limiting and other and further objects, features and advantages of the invention will be apparent from the following detailed description of the presently preferred embodiments of the invention and from the claims.

Brief Description of the Drawings

Figure 1 shows SEAP serum concentrations at day 7 post injection of SEAP pDNA/empty DNA mixtures in the tibialis cranialis muscle of CD-1 mice with electroporation. Various SEAP pDNA amounts and empty pDNA excess (relative to the coding pDNA) were administered.

Figure 2 shows SEAP serum concentrations at day 7 post injection of naked SEAP pDNA or SEAP pDNA/anionic polymer mixtures in the tibialis cranialis muscle of CD-1 mice with electroporation and DNA concentration of 2.5 micrograms in 50 microliters (half this dose per leg). The concentration of the anionic polymer in the injected solution varied as indicated on the graph.

Figure 3 shows SEAP serum concentrations at day 7 post injection of naked SEAP pDNA or SEAP pDNA/anionic polymer mixtures in the tibialis cranialis muscle of CD-1 mice with electroporation and the amount of SEAP pDNA administered per animal was regularly (unless mentioned) 25 micrograms in 50 microliters (half this dose per leg).

Figure 4 shows SEAP serum concentrations at day 7 post injection of naked SEAP pDNA or SEAP pDNA/anionic polymer mixtures in the gastrocnemius muscle of CD-1 mice and

electroporation of the tissue. The concentration of the anionic polymer in the injected solution varied as indicated on the graph.

Figure 5 shows SEAP serum concentrations at day 7 as a function of the amount of SEAP pDNA injected in different formulations as indicated: **A** in the tibialis cranialis muscle of CD-1 mice; **B** in the gastrocnemius muscle of CD-1 mice comparing either naked SEAP pDNA or a mixture of SEAP pDNA and a poly-L-glutamic acid at 6.0 mg/ml.

Figure 6 shows luciferase expression after direct intramyocardial injection of plasmid DNA formulated in saline versus poly-glutamic acid.

Figure 7 shows hF.IX serum concentrations at day 7 post injection of naked hF.IX pDNA or hF.IX pDNA/poly-L-glutamic acid mixtures in the tibialis muscle of C57BL/6 mice and electroporation of the tissue. The concentration of the anionic polymer in the injected solution varied as indicated on the graph.

Figure 8 shows hF.IX expression in plasma of immune deficient (SCID beige) mice.

Figure 9 depicts the immunohistology and fiber-type of hF.IX expressing myocytes in SCID mouse muscle.

Figure 10 A depicts plasma hF.IX levels determined by ELISA in dogs following intramuscular injection of plasmid augmented by electroporation at different numbers of sites. Values are means ± SEM with n = 3 for each group. **Figure 10B** shows a western blot of purified hF.IX using treated animal serum as the primary antibody. Lane A, molecular marker; lane B, negative control serum; lane C, positive control (canine serum spiked with rabbit anti-hF.IX antibodies; lane D, serum

from a female dog from the 6 injection group (peak expression hF.IX 35.71 ng/ml); lane E, serum from a male dog from the 12 injection group (peak hF.IX expression 47.9 ng/ml).

Figure 11 depicts the duration of retention of the mouse EPO plasmid DNA following delivery by electroporation using saline and poly-L-glutamic acid formulations.

Figure 12 depicts EPO expression and hematocrit in mice following delivery of the mouse EPO gene by electroporation using saline and poly-L-glutamic acid formulations.

Figure 13 depicts the results of the EPO expression in mice following delivery of the mouse EPO gene by electroporation using saline and poly-L-glutamic acid formulations over a three month time frame.

Figure 14 depicts a comparison of hINFα gene expression after delivery in saline versus polyglutamate. **A** depicts the results using a 50 microgram dose of plasmid DNA while **B** depicts the results of administration of a 5 microgram dose of plasmid DNA.

Figure 15 shows the ability of poly-L-glutamate and poloxamer formulations to protect DNA from nuclease degradation. Panel A represents a DNA in saline formulation; Panel B represents DNA formulated in 5% Pluronic F68; Panel C represents DNA formulated in 6 mg/ml poly-L-glutamate. Lane A, negative control of plasmid DNA without DNase; lane B, positive control of plasmid DNA and DNase mixed 1:1; lane C, DNase diluted 1:1; lane D, DNase diluted 1:10; lane E, DNase diluted 1:100; lane F, DNase diluted 1:1,000; lane G, DNase diluted 1:10,000.

Figure 16 depicts the results of long term biological stability of plasmid DNA encoding SEAP formulated in 6 mg/ml

poly-L-glutamate under different storage conditions. A, lyophilization and storage at 4°C. for 105 days; B, freezing of a liquid formulation with storage at -20°C. for 105 days; C, liquid storage at 4°C. for 105 days; D, liquid storage at room temperature for 105 days; E, liquid storage at 37°C. for 105 days; F, liquid storage at 50°C. for 8 days; G, liquid formulation subject to freeze/thawing; H, fresh DNA formulated on poly-L-glutamate; I, fresh DNA without poly-L-glutamate.

Figure 17 depicts the plasmid map for pFN0945, an expression plasmid carrying the gene for hF.IX. The sequence of the complete plasmid is disclosed as SEQ. ID. NO. 3.

Figure 18 depicts the plasmid map for pFN1645, an expression plasmid carrying an codon optimized gene for hF.IX. The sequence of the complete plasmid is disclosed as SEQ. ID. NO. 4.

Figure 19 depicts the plasmid map for pEP1403, an expression plasmid carrying the mouse erythropoietin gene. The sequence of the complete plasmid is disclosed as SEQ. ID. NO. 2.

Figure 20 depicts the plasmid map for pIF0921, an expression plasmid carrying the human interferon alpha gene. The sequence of the complete plasmid is disclosed as SEQ. ID. NO. 1.

Detailed Description of the Preferred Embodiments

The delivery and expression of sequences encoded on a vector in eukaryotic cells, particularly *in vivo* in a mammal, depends on a variety of factors including transfection efficiency and lifetime of the coding sequence within the

transfected cell. Thus, a number of methods are reported for accomplishing such delivery.

A non-viral gene medicine is composed of three major elements: *i)* a nucleic acid encoding a gene product (e.g., a therapeutic protein), *ii)* a plasmid-based expression system, and *iii)* a synthetic gene delivery system. These products are intended to have low toxicity due to the use of synthetic components for gene delivery (minimizing for instance the risks of immunogenicity generally associated with viral vectors) and non-integrating plasmids for gene expression. Since no integration of plasmid sequences into host chromosomes has been reported *in vivo* to date, they should neither activate oncogenes nor inactivate tumor suppressor genes. This built-in safety with non-viral systems contrasts with the risks associated with the use of most viral vectors. As episomal systems residing outside the chromosomes, plasmids have defined pharmacokinetics and elimination profiles, leading to a finite duration of gene expression in target tissues.

Formulating the nucleic acid with anionic polymers as disclosed below is particularly desirable because they enhance transfection and expression of the nucleic acid, protect the nucleic acid from degradation, and are completely biodegradable. In addition, because formulating the nucleic acid with anionic polymers results in more efficient transfection, lower amounts of DNA may be used. By biodegradable, it is meant that the anionic polymers can be metabolized or cleared by the organism *in vivo* without any or minimal toxic effects or side effects. The term "anionic polymers" means polymers having a repeating subunit which includes, for example, an ionized carboxyl, phosphate or

sulfate group having a net negative charge at neutral pH. Examples of the anionic polymers include poly-amino acids (such as poly-glutamic acid, poly-aspartic acid and combinations thereof), poly nucleic acids, poly acrylic acid, poly galacturonic acid, and poly vinyl sulfate. In the case of polymeric acids, the polymer will typically be utilized as the salt form.

Efforts have been made to enhance the delivery of plasmid DNA to cells by physical means including electroporation, sonoporation and pressure. Injection by electroporation is a modern technique that involves the application of a pulsed electric field to create transient pores in the cellular membrane without causing permanent damage to the cell and thereby allows for the introduction of exogenous molecules. This technique has been used widely in research laboratories to create hybridomas and is now being applied to gene transfer approaches for therapy. By adjusting the electrical pulse generated by an electroporetic system, nucleic acid molecules can find their way through passageways or pores in the cell that are created during the procedure. U. S. Patent 5,704,908 describes an electroporation apparatus for delivering molecules to cells at a selected location within a cavity in the body of a patient. (U. S. Patent 5,704,908, including any drawings contained therein, is hereby incorporated by reference as if fully set forth herein.)

The use of electroporetic methods to deliver genes suspended in saline into rabbit and porcine arteries as models to treat coronary and peripheral vascular disease has been discussed at the 3rd US-Japan Symposium on Drug Delivery (D. B. Dev, J. J. Giordano and D. L. Brown, Maui, Hawaii, December

17-22, 1995). The ability to target and express the lacZ reporter gene suspended in saline to various depths of the dermis region in hairless mice has been described in the article "Depth-Targeted Efficient Gene delivery and Expression in the skin by Pulsed Electric Fields: An approach to Gene Therapy of Skin Aging and Other Diseases" (Zhang et al., Biochemical and Biophysical Research Communications 220, 633-636 (1996)). A mammalian expression plasmid for the lacZ gene in saline has been injected into the internal carotid artery of rats whose brain tumors had been electroporated between two electrodes. The gene was reported to be expressed in the tumor cells three days after plasmid injection and furthermore, lacZ activity was reported to be isolated only to the tissues and cells targeted (Nishi, et al., Cancer Research 56, 1050-1055, March 1, 1996).

Formulations for electroporation are described in U.S. Patent Application Serial No. 09/322,602, which is incorporated herein by reference in its entirety, including any drawings. By adjusting the electrical pulse generated by an electroporetic system, nucleic acid molecules can find their way in the cell through passageways or pores that are created during the procedure.

Previously, treatment of hemophilia B by non-viral methods was not been possible because only low and variable levels of gene expression were achieved. Recently, the use of electroporation *in vivo* was shown to produce consistent high levels of gene expression in muscle, liver, skin, solid tumors and testis following direct injection of plasmid into these tissues (Titomirov, A.V., et al. (1991) *Biochim Biophys Acta* 1088: 131-134; Muramatsu, T., et al. (1997) *Biochem Biophys*

Res Commun 233: 45-49; Suzuki, T., et al. (1998) *FEBS Lett* 425: 436-440; Aihara, H. and Miyazaki, J. (1998) *Nat Biotechnol* 16: 867-870; Mir, L.M., et al. (1998) *C R Acad Sci III* 321: 893-899; Rizzuto, G., et al. (1999) *Proc Natl Acad Sci U S A* 96: 6417-6422; Goto, T., et al (2000) *Proc Natl Acad Sci U S A* 97:354-359; Somiari, S., et al. (2000) *Mol Ther* 2:178-187). In mice, electroporation of plasmid DNA in saline was used to achieve circulating levels of hF.IX that were 2% of normal and maintained for at least 2 months (Bettan, M., et al. (2000) *Mol Ther* 2:204-210). The present application discloses novel plasmid formulations for electroporation that achieve four goals: (1) therapeutically significant levels of proteins *in vivo*, (2) persistent expression of the transgene, (3) re-administration of formulated plasmid to obtain levels comparable to the initial levels and (4) therapeutically significant levels in large animals.

The delivery of a formulated DNA according to the present invention by the use of pulse voltage delivery device represents a novel approach to gene delivery. In particular, the the preferred embodiment employing anionic amino acid polymers or poly-amino acids were able to substantially increase the expression of introduced genes by electroporation when compared with saline. The poly-amino acids also have the advantage over prior formulations by being completely biodegradable. The preferred embodiment also provides the advantage of allowing the uptake of formulated nucleic acid molecules (i.e., nucleic acid molecules in the compositions of the invention) by specifically targeted cells and cell lines, as well as uptake by multiple cell lines as desired. Injecting

formulated nucleic acid molecules by pulse voltage delivery methods results in the formulated nucleic acid molecules gaining access to the cellular interior more directly through the destabilization of the cell wall and/ or by the formation of pores as a result of the electroporetic process. Furthermore, in certain instances multiple cell lines can be targeted, thus allowing contact to many more cell types than in conventional needle injection. Thus, the present invention provides an enhanced delivery of nucleic acid molecules and also provides a more efficient gene delivery system which may be used to generate an immune response, express a therapeutic gene, modulate aspects of the cell cycle or cell physiology, or provide a method to achieve other gene delivery related therapeutic methods such as anti-tumor therapy.

The term "poly-L-glutamic acid" is used interchangeably herein with "poly-L-glutamic acid, sodium salt", "sodium poly-L-glutamate" and "poly-L-glutamate." "Poly-L-glutamate" refers to the sodium salt of poly-L-glutamic acid. Although the L stereoisomer of polyglutamic acid was found to be particularly useful, the other stereoisomer or racemic mixtures of isomers are within the scope of the invention. The present invention contemplates that other salts of anionic amino acid polymers may be equally suitable.

The term "anionic amino acid polymers" means polymeric forms of a given anionic amino acid such as, for example, poly-glutamic acid or poly-aspartic acid. The present invention contemplates that polymers formed of a mixture of anionic amino acids, such as for example glutamic acid and aspartic acid, may be equally suitable.

By "delivery" or "delivering" is meant transportation of nucleic acid molecules to desired cells or any cells. The nucleic acid molecules may be delivered to multiple cell lines, including the desired target. Delivery results in the nucleic acid molecules coming in contact with the cell surface, cell membrane, cell endosome, within the cell membrane, nucleus or within the nucleus, or any other desired area of the cell from which transfection can occur within a variety of cell lines which can include but are not limited to; tumor cells, epithelial cells, Langerhan cells, Langhans' cells, littoral cells, keratinocytes, dendritic cells, macrophage cells, Kupffer cells, muscle cells, lymphocytes and lymph nodes. Preferably, the composition of the invention is delivered to the cells by electroporation and the nucleic acid molecule component is not significantly sheared upon delivery, nor is cell viability directly effected by the pulse voltage delivery process.

By "nucleic acid" is meant both RNA and DNA including: cDNA, genomic DNA, plasmid DNA or condensed nucleic acid, nucleic acid formulated with cationic lipids, nucleic acid formulated with peptides, cationic polymers, RNA or mRNA. In a preferred embodiment, the nucleic acid administered is a plasmid DNA which constitutes a "vector". The nucleic acid can be, but is not limited to, a plasmid DNA vector with a eukaryotic promoter which expresses a protein with potential therapeutic action, such as, for example; hGH, VEGF, EPO, IGF-I, TPO, Factor IX, IFN-α, IFN-β, IL-2, IL-12, or the like.

As used herein, the term a "plasmid" refers to a construct made up of genetic material (i.e., nucleic acids). It includes genetic elements arranged such that an inserted

coding sequence can be transcribed in eukaryotic cells. Also, while the plasmid may include a sequence from a viral nucleic acid, such viral sequence preferably does not cause the incorporation of the plasmid into a viral particle, and the plasmid is therefore a non-viral vector. Preferably, a plasmid is a closed circular DNA molecule. The enhancer/promoter region of an expression plasmid will determine the levels of expression. Most of the gene expression systems designed for high levels of expression contain the intact human cytomegalovirus (CMV) immediate early enhancer/promoter sequence. However, down-regulation of the CMV promoter over time has been reported in tissues. The hypermethylation of the CMV promoter, as observed when incorporated into retroviral vectors, has not been observed for episomal plasmids *in vivo*. Nevertheless, the CMV promoter silencing could be linked to its sensitivity to reduced levels of the transcription factor NF-κB. The activity of the CMV promoter has also been shown to be attenuated by various cytokines including interferons (α and β), and tumor necrosis factor (TNF-α). In order to prolong expression *in vivo* and ensure specificity of expression in desired tissues, tissue-specific enhancer/promoters have been incorporated in expression plasmids. The chicken skeletal alpha actin promoter has been shown to provide high levels of expression (equivalent to the ones achieved with a CMV-driven construct) for several weeks in non-avian striated muscles.

Additional genetic sequences in the expression plasmids can be added to influence the stability of the messenger RNA (mRNA) and the efficiency of translation. The 5′ untranslated region (5′ UTR) is known to effect translation and it is located between the cap site and the initiation codon. The 5′

UTR should ideally be relatively short, devoid of strong secondary structure and upstream initiation codons, and should have an initiation codon AUG within an optimal local context. The 5' UTR can also influence RNA stability, RNA processing and transcription. In order to maximize gene expression by ensuring effective and accurate RNA splicing, one or more introns can be included in the expression plasmids at specific locations. The possibility of inefficient and/or inaccurate splicing can be minimized by using synthetic introns that have idealized splice junction and branch point sequences that match the consensus sequence. Another important sequence within a gene expression system is the 3' untranslated region (3' UTR), a sequence in the mRNA that extends from the stop codon to the poly(A) addition site. The 3' UTR can influence mRNA stability, translation and intracellular localization. The skeletal muscle α-actin 3' UTR has been shown to stabilize mRNA in muscle tissues thus leading to higher levels of expression as compared to other 3' UTR. This 3' UTR appears to induce a different intracellular compartmentalization of the produced proteins, preventing the effective trafficking of the proteins to the secretory pathway and favoring their perinuclear localization.

One of the attractive features of plasmid expression systems is the possibility to express multiple genes from a single construct. These multivalent systems may find applications in the expression of heterodimeric proteins, such as antibodies, or in the *in vivo* production of multiple antigens to generate a potent immune response for genetic vaccination. In cancer immunotherapy, the co-expression of co-

stimulatory molecules with a variety of cytokines may also lead to enhanced responses.

The term "vector" as used herein refers to a construction including genetic material designed to direct transformation of a targeted cell. A vector contains multiple genetic material, preferably contiguous fragments of DNA or RNA, positionally and sequentially oriented with other necessary elements such that the nucleic acid can be transcribed and when necessary translated in the transfected cells. The "vector" preferably is a nucleic acid molecule incorporating sequences encoding therapeutic product(s) as well as, various regulatory elements for transcription, translation, transcript stability, replication, and other functions as are known in the art. The vector preferably allows for production of a product encoded for by a nucleic acid sequence contained in the vector. For example, expression of a particular growth factor protein encoded by a particular gene. A "DNA vector" is a vector whose native form is a DNA molecule. A "viral vector" is a vector whose native form is as the genomic material of a viral particle.

The term "transfection" as used herein refers to the process of introducing DNA (e.g., formulated DNA expression vector) into a cell, thereby, allowing cellular transformation. Following entry into the cell, the transfected DNA may: (1) recombine with that of the host; (2) replicate independently as a plasmid or temperate phage; or (3) be maintained as an episome without replication prior to elimination.

As used herein, "transformation" relates to transient or permanent changes in the characteristics (expressed phenotype) of a cell induced by the uptake of a vector by that cell.

Genetic material is introduced into a cell in a form where it expresses a specific gene product or alters the expression or effect of endogenous gene products. Transformation of the cell may be associated with production of a variety of gene products including protein and RNA. These products may function as intracellular or extracellular structural elements, ligands, hormones, neurotransmitters, growth regulating factors, enzymes, chemotaxins, serum proteins, receptors, carriers for small molecular weight compounds, drugs, immunomodulators, oncogenes, cytokines, tumor suppressors, toxins, tumor antigens, antigens, antisense inhibitors, triple strand forming inhibitors, ribozymes, or as a ligand recognizing specific structural determinants on cellular structures for the purpose of modifying their activity. This list is only an example and is not meant to be limiting.

A "gene product" means products encoded by the vector. Examples of gene products include mRNA templates for translation, ribozymes, antisense RNA, proteins, glycoproteins, lipoproteins, phosphoproteins and polypeptides. The nucleic acid sequence encoding the gene product may be associated with a targeting ligand to effect targeted delivery.

"Uptake" means the translocation of the vector from the extracellular to intracellular compartments. This can involve receptor-mediated processes, fusion with cell membranes, endocytosis, potocytosis, pinocytosis or other translocation mechanisms. The vector may be taken up by itself or as part of a complex.

Administration as used herein refers to the route of introducing the compositions of the invention into the body of cells or organisms. Administration includes the use of

electroporetic methods as provided by a pulse voltage device to targeted areas of the mammalian body such as the muscle cells and the lymphatic cells in regions such as the lymph nodes. Administration also includes intradermal, intra-tumoral and subcutaneous administration.

A "therapeutically effective amount" of a composition is an amount that is sufficient to cause at least temporary relief or improvement in a symptom or indication of a disease or condition. Thus, the amount is also sufficient to cause a pharmacological effect. The amount of the composition need not cause permanent improvement or improvement of all symptoms or indications.

The term "pulse voltage device", or "pulse voltage injection device" as used herein relates to an apparatus that is capable of causing or causes uptake of nucleic acid molecules into the cells of an organism by emitting a localized pulse of electricity to the cells, thereby causing the cell membrane to destabilize and result in the formation of passageways or pores in the cell membrane. It is understood that conventional devices of this type are calibrated to allow one of ordinary skill in the art to select and/or adjust the desired voltage amplitude and/or the duration of pulsed voltage and therefore it is expected that future devices that perform this function will also be calibrated in the same manner. The type of injection device is not considered a limiting aspect of the present invention. The primary importance of a pulse voltage device is, in fact, the capability of the device to facilitate delivery of compositions of the invention into the cells of an organism. The pulse voltage injection device can include, for example, an electroporetic apparatus as described

in U.S. Patent 5,439,440, U.S. Patent 5,704,908 or U.S. Patent 5,702,384 or as published in PCT WO 96/12520, PCT WO 96/12006, PCT WO 95/19805, and PCT WO 97/07826, all of which are incorporated herein by reference in their entirety.

The term "apparatus" as used herein relates to the set of components that upon combination allow the delivery of compositions of the invention into the cells of an organism by pulse voltage delivery methods. The apparatus of the invention can be a combination of a syringe or syringes, various combinations of electrodes, devices that are useful for target selection by means such as optical fibers and video monitoring, and a generator for producing voltage pulses which can be calibrated for various voltage amplitudes, durations and cycles. The syringe can be of a variety of sizes and can be selected to inject compositions of the invention at different delivery depths such as to the skin of an organism such as a mammal, or through the skin.

The term "organism" as used herein refers to common usage by one of ordinary skill in the art. The organism can include microorganisms, such as yeast or bacteria, plants, birds, reptiles, fish or mammals. The organism can be a companion animal or a domestic animal. Preferably the organism is a mammal and is therefore any warmblooded organism. More preferably the mammal is a human.

The term "companion animal" as used herein refers to those animals traditionally treated as "pets" such as for example, dogs, cats, horses, birds, reptiles, mice, rabbits, hamsters, and the like. The term "domestic animal" as used herein refers to those animals traditionally considered domesticated, where animals such as those considered "companion

animals" are included along with animals such as, pigs, chickens, ducks, cows, goats, lambs, and the like.

By "prolong the localized bioavailability of a nucleic acid" is meant that a nucleic acid when administered to an organism in a composition comprising such a compound will be available for uptake by cells for a longer period of time than if administered in a composition without such a compound, for example when administered in a formulation such as a saline solution. This increased availability of nucleic acid to cells could occur, for example, due to increased duration of contact between the composition containing the nucleic acid and a cell or due to protection of the nucleic acid from attack by nucleases. The compounds that prolong the localized bioavailability of a nucleic acid are suitable for internal administration.

By "suitable for internal administration" is meant that the compounds are suitable to be administered within the tissue of an organism, for example within a muscle or within a joint space, intradermally or subcutaneously. Other forms of administration which may be utilized are topical, oral, pulmonary, nasal and mucosal; for example, buccal, vaginal or rectal. Properties making a compound suitable for internal administration can include, for example, the absence of a high level of toxicity to the organism as a whole.

By "solutions" is meant water soluble polymers and/or surfactants in solution with nucleic acids.

Polymeric formulations for plasmid delivery to muscle

The present invention provides polymeric formulations that address problems associated with injection of nucleic

acids suspended in saline. Unformulated (naked nucleic acid molecules) plasmids suspended in saline have poor bioavailability in muscle due to rapid degradation of plasmid by extracellular nucleases. One possible approach to overcome the poor bioavailability is to protect plasmid from rapid nuclease degradation by, for example, condensing the plasmid with commonly used cationic complexing agents. However, due to the physiology of the muscle, the use of rigid condensed particles containing plasmid for efficient transfection of a larger number of muscle cells has not been successful to date. Cationic lipid and polylysine plasmid complexes do not cross the external lamina to gain access to the caveolae and T tubules (Wolff, J.A., et al., 1992, *J. Cell. Sci.* 103:1249-1259).

Thus, the invention increases the bioavailability of plasmid in muscle by: protecting plasmid from rapid extracellular nuclease degradation; dispersing and retaining intact plasmid in the muscle and/or tumor; and facilitating the uptake of plasmid by muscle and/ or tumor cells. A specific method of accomplishing this, which preferably is used in conjunction with pulse voltage delivery, is the use of anionic polymers.

Administration

Administration as used herein refers to the route of introduction of a plasmid or carrier of DNA into the body. Administration can be directly to a target tissue or by targeted delivery to the target tissue after systemic administration. In particular, the present invention can be used for treating conditions by administration of the

formulation to the body in order to establish controlled expression of any specific nucleic acid sequence within tissues at certain levels that are useful for gene therapy.

The preferred means for administration of vector (plasmid) and use of formulations for delivery are described above. The preferred embodiments are by pulse voltage delivery to cells in combination with needle or needle free injection, or by direct applied pulse voltage wherein the electroporation device's electrodes are pressed directly against the targeted tissue or cells, such as for example epidermal cells, and the vector is applied topically before or after pulse application and delivered through and or to the cells.

The route of administration of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation for each vector construct used will focus on vector delivery with regard to the particular targeted tissue, the pulse voltage delivery parameters, followed by demonstration of efficacy. Delivery studies will include uptake assays to evaluate cellular uptake of the vectors and expression of the DNA of choice. Such assays will also determine the localization of the target DNA after uptake, and establishing the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Muscle cells have the unique ability to take up DNA from the extracellular space after simple injection of DNA particles as a solution, suspension, or colloid into the muscle. Expression of DNA by this method can be sustained for several months.

The chosen method of delivery should result in expression of the gene product encoded within the nucleic acid cassette at levels that exert an appropriate biological effect. The rate of expression will depend upon the disease, the pharmacokinetics of the vector and gene product, and the route of administration, but should be in the range 0.001-100 mg/kg of body weight/day, and preferably 0.01-10 mg/kg of body weight/day. This level is readily determinable by standard methods. It could be more or less depending on the optimal dosing. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon the disease, delivery vehicle, and efficacy data from clinical trials.

DNA Injection Variables

The level of gene delivery and expression or the intensity of an immune response achieved with the present invention can be optimized by altering the following variables. The variables are: the formulation (composition, plasmid topology), the technique and protocol for injection (area of injection, duration and amplitude of voltage, electrode gap, number of pulses emitted, type of needle arrangement, pre-injection-pulsed or post-injection-pulsed cells, state of muscle, state of the tumor), and, the pretreatment of the muscle with myotoxic agents. An immune response can be measured by, but is not limited to, the amount of antibodies produced for a protein encoded and expressed by the injected nucleic acid molecule.

Other injection variables that can be used to significantly affect the levels of proteins, antibodies and/or

cytotoxic T-lymphocytes produced in response to the protein encoded by the formulated nucleic acid molecule provided by the pulse voltage injection method of the present invention are the state of the muscle being injected and injection technique. Examples of the variables include muscle stimulation, muscle contraction, muscle massage, delivery angle, and apparatus manipulation. Massaging the muscle may force plasmid out of the muscle either directly or via lymphatic drainage. By altering the depth of penetration and/or the angle at which the pulse voltage device is placed in relation to muscle fibers the present invention improves the plasmid distribution throughout the injection area that subsequently increases the antibody response to the protein which is encoded and expressed by the plasmid.

Nucleic acid based therapy

The present invention can be used to deliver nucleic acid vaccines in a more efficient manner than is conventionally done at the present time. Nucleic acid vaccines, or the use of plasmid encoding antigens or therapeutic molecules such as Human Growth Hormone, has become an area of intensive research and development in the last half decade. Comprehensive reviews on nucleic acid based vaccines have been published (M.A. Liu, et al.(Eds.), 1995, *DNA Vaccines: A new era in vaccinology*, Vol. 772, Ann. NY. Acad. Sci., New York; Kumar, V., and Sercarz, E., 1996, *Nat. Med.* 2:857-859; Ulmer, J.B., et al., (Eds.) *Current Opinion in Immunology*; 8:531-536. Vol. 772, Ann. NY. Acad. Sci., New York). Protective immunity in an animal model using plasmid encoding a viral protein was first observed in 1993 by Ulmer et al. (Ulmer, J.B., et al., 1993, *Science*

259:1745-1749). Since then, several studies have demonstrated protective immunity for several disease targets and human clinical trials have been started.

Many disease targets have been investigated. Examples include antigens of *Borrelia burgdorferi*, the tick-borne infectious agent for Lyme disease (Luke et al., *J. Infect. Dis.* 175:91-97, 1997), human immunodeficiency virus-1, (Letvin et al., *Proc. Nat. Acad. Sci. USA* 94:9378-9383, 1997), B cell lymphoma (Syrengelas et al., *Nature Medicine*. 2:1038-41, 1996), Herpes simplex virus (Bourne et al., *J. Infectious dis.* 173:800-807, 1996), hepatitis C virus (Tedeschi et al., *Hepatology* 25:459-462, 1997), rabies virus (Xiang et al., *virology*, 209:569-579, 1995), *Mycobacterium tuberculosis* (Lowrie in Genetic Vaccines and Immunotherapeutic Strategies CA Thibeault, ed. Intl Bus Comm, Inc., Southborough, MA 01772 pp. 87-122, 1996), and *Plasmodium falciparum* (Hoffman et al., *Vaccine* 15:842-845, 1997). Additionally, nucleic acid based treatment for reducing tumor-cell immunogenicity, growth, and proliferation is indicative of gene therapy for diseases such as tumorigenic brain cancer (Fakhrai et al., Proc. Natl. Acad. Sci., 93:2909-2914, 1996).

An important goal of gene therapy is to affect the uptake of nucleic acid by cells, thereby causing an immune response to the protein encoded by the injected nucleic acid. Nucleic acid based vaccines are an attractive alternative vaccination strategy to subunit vaccines, purified viral protein vaccines, or viral vector vaccines. Each of the traditional approaches has limitations that are overcome if the antigen(s) is expressed directly in cells of the body. Furthermore, these traditional vaccines are only protective in a strain-specific

fashion. Thus, it is very difficult, and even impossible using traditional vaccine approaches to obtain long lasting immunity to viruses that have several sera types or viruses that are prone to mutation.

Nucleic acid based vaccines offer the potential to produce long lasting immunity against viral epitopes that are highly conserved, such as with the nucleoprotein of viruses. Injecting plasmids encoding specific proteins by the present invention results in increased immune responses, as measured by antibody production. Thus, the present invention includes new methods of providing nucleic acid vaccines by delivering a formulated nucleic acid molecule with a pulse voltage device as described herein.

The efficacy of nucleic acid vaccines is enhanced by one of at least three methods: (1) the use of delivery systems to increase the stability and distribution of plasmid within the muscle, (2) by the expression (or delivery) of molecules to stimulate antigen presentation/transfer, or (3) by the use of adjuvants that may modulate the immune response.

Diseases and Conditions for Intramuscular Plasmid Delivery

The present invention described herein can be utilized for the delivery and expression of many different coding sequences. The coding sequences may be used to ameliorate the effects of inborn errors of metabolism, genetic deficiencies of certain necessary proteins, acquired metabolic and regulatory imbalances and disordered cellular regulation such as with cancer. The coding sequence containing composition preferably is administered by pulsed voltage delivery and may

require, as needed, exposure of the tissue to be treated by surgical means as determined by a certified professional.

EXAMPLES

The following examples are offered by way of illustration and are not intended to limit the scope of the invention in any manner. One of ordinary skill in the art would recognize that the various molecules and/ or amounts disclosed in the examples could be adjusted or substituted. It would also be recognized that the delivery targets and/ or amounts delivered in the examples could be adjusted or substituted by selecting different muscles for injection, injection into tumors or nodes, or increasing or decreasing the duration of pulse time or alternating the pulse application from pre-injection to post-injection.

Preparation of Formulations

Formulations were made by aliquoting appropriate volumes of sterile stock solutions of water, plasmid, polymer, buffer and/or 5M NaCl to obtain a final plasmid in an isotonic solution. The total plasmid concentration of all formulations was measured by UV absorption at 260 nm. The osmotic pressure of selected formulations was measured using a Fiske One-Ten Micro-Sample Osmometer (Fiske Associates; Norwood, MA). The percentage of supercoiled plasmid was measured using 1% agarose gel electrophoresis followed by fluorimaging.

Plasmids were formulated in 5 - 10 mM Tris, pH 7.5 or saline (150 mM NaCl) or mixed with a polymer in isotonic saline. Plasmid used for injection was formulated with various polymers in an isotonic saline solution. Typically, the concentration of plasmid was 1-2 mg/ml in saline, or formulated

with polyvinylpyrrolidone (PVP, 5%) or 6 mg/ml poly-L-glutamate (Sigma, St Louis, MO) in saline.

Anionic polymers included poly-L-glutamic acid (p-L-Glu), sodium salt, of various molecular weights (degree of polymerization (DP) of 9 (Sigma P1943), degree of polymerization of 10 (Sigma P1818), 2-15 kDa (Sigma P4636), 15-50 kDa (Sigma P4761) and 50-100 kDa (Sigma P4886)), poly-D-glutamic acids (p-D-Glu) of 15-50 (Sigma P4033) and 50-100 kDa (Sigma 4637), poly-L-aspartic acid (p-L-Asp), sodium salt, of 2-15 (Sigma P5387) and 15-50 kDa (Sigma P6762) and poly-acrylic acid (pAA), sodium salt, of 5 and 60 kDa. The polyamino acids were purchased from Sigma (St. Louis, MO), while the poly(acrylic acid) was acquired from Fluka (Switzerland).

The DNA/anionic polymer formulations were preferably prepared by aliquoting appropriate volumes of sterile stock solutions of plasmid, anionic polymer and 5M NaCl to obtain selected final plasmid and anionic polymer concentrations. The anionic polymer was added to the DNA solution prior to adding salt for tonicity adjustment. Thus, poly-L-glutamate formulations are preferably prepared by combining an aqueous stock solution of sodium poly-L-glutamate (sodium salt of poly-L-glutamic acid) with a stock solution of purified plasmid DNA in saline or up to 10mM Tris, pH 7.5. After the poly-L-glutamic acid and DNA are combined, the solution is adjusted to a final concentration of 150mM NaCl by addition of a stock solution of 5M NaCl.

The osmolality of each formulation was measured using a Fiske One-Ten Micro-Sample Osmometer (Fiske Associate, Norwood MA). Formulations were also characterized by measuring the

optimal density at 260 and 280 nm, and by determining plasmid conformation on a 1% agarose gel.

Stability Test For Plasmid In The Formulation

For the analysis of pDNA stability in the formulation, 50 ng of formulated pDNA with 5 microliters of tracking dye was loaded into 1% agarose gel in 1% tris-acetate-EDTA (TAE) buffer and run the gel at 100 volts for 1-2 hours. The gel was then stained with SYBR Green II (Molecular Probes, Inc.) for 20 minutes. The stained gel was washed with water and % of supercoiled and open circled DNA was determined using a Fluorinate (Molecular Dynamics Co., Sunnyvale, CA).

Elisa protocol

High affinity assay plates were coated with antigen diluted in PBS (50 microliters/well) and placed at 4°C overnight. After allowing plate(s) to come to room temperature, all wells were blocked with 200 microliters/well of 4% BSA/4% NGS solution made in 1X PBS/Tween20 for 1 hr at 37 ° C. Add serum samples (50 microliters/well at a starting dilution of 1:100 in 4% BSA/4% NGS/PBS/Tween20, in duplicate) and incubate for 1-2 hours at 37 ° C. Wash plate(s) with PBS/Teen 20 and add 50 microliters/well of HRP-conjugated secondary, diluted in 1% BSA, and incubate at 37°C for 1 hour. Wash plate(s) with PBS/Teen 20 and add 100 microliters/well of TMB soluble reagent. Incubate at room temperature for 10 minutes and stop the reaction by adding 50 microliters/well of 0.2M H_2SO_4. Read plate(s) at 450 nm.

Plasmids

Plasmids pAP1166 and pFN0945 (SEQ. ID. NO. 3) containing a CMV enhancer-promoter and either a human placental secreted alkaline phosphatase reporter gene (SEAP) (pAP1166) or the coding region of hF.IX (pFN0945 SEQ. ID. NO. 3) were manufactured and purified at Valentis, Inc. The plasmid map of pFN0945 is shown in Figure 17. Human factor IX (hF.IX) plasmid was prepared by inserting a synthetic coding sequence in which rare codons were converted to prevalent ones and potential cryptic splice sites were abrogated (Oberon Technologies Inc., Alameda, CA). The hF.IX coding sequence was inserted into the Valentis plasmid backbone containing a 107 bp 5'UTR, a 117 bp synthetic intron, the human growth hormone polyadenylation signal, a PUC12 origin of replication and a kanamycin resistance gene. The hF.IX gene was driven by the CMV enhancer/promoter. Plasmids were grown in *Escherichia coli* DH5α and were purified using a proprietary method involving alkaline lysis and chromographic methods (Abruzzese, R.V., et al. (1999) *Hum Gene Ther* 10:1499-1507, incorporated herein by reference). The human secreted alkaline phosphatase (SEAP) and human erythropoietin plasmids were identical to the hF.IX plasmid except for the coding region.

Experimental Animals

Male C57BL/6 mice (19-21 g), male CD-1 mice (29-31g), male C.B-17/lcrCrl-scid-bgBR (SCID BEIGE) mice (7 weeks of age) and female C57BL/6 mice (7-8 weeks) were obtained from Charles River Laboratories and were acclimatized for a 3-7 day period in a 12 hour light-dark cycle at 23°C/40 % RH in accordance with state and federal guidelines. Food (Purina rodent chow)

and water were provided *ad libitum*. The animals were housed in hepa-filtered caging units (4 mice per isolator) with sterilized bedding food and water. Cage exchange and all manipulations with the SCID mice were performed in a laminar flow hood. Animals were anesthetized via intraperitioneal (IP) injection with a combination anesthesia (Ketamine, Xylazine and Acepromazine) at a dose of 1.8-2.0 mL/kg (mice). Beagle dogs (Harlan, Indianapolis, IN) were maintained at Stillmeadow, Inc. (Sugarland, TX) in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Animal Injections

After anesthestia, hind limbs were shaved and scrubbed with betadine followed by 70% ethanol. 10 microliters of the formulation was injected with 10 micrograms of formulated plasmid using a 0.3-ml insulin syringe with a 28-gauge, 0.5 needle (Becton Dickinson, Granklin Lake, NJ). The injected volumes in mice were 25 microliters and 50 microliters in the cranial tibialis and gastrocnemius, respectively. Where indicated, seven days after formulation injection, the animals were sacrificed by CO_2 asphyxiation and the tibialis anterior muscles was harvested, quickly immersed in liquid nitrogen, and lyophilized overnight. The dried muscles were used or stored at -80°C for further determination of reporter gene activity.

Device and Dosing Regimens

Plasmid formulated at the required dose was administered in rodents by longitudinal injection in both tibialis cranialis or in both gastrocnemius muscles (bilateral administration). By holding the entire lower leg between the caliper electrodes good "electrotransfection" could be obtained. Approximately,

two minutes after injection, an electric field was applied in the form of 2 square wave pulses (one per second) of 25 millisecond ("ms") each and 375 V/cm delivered by an Electro Square Porator (T820, BTX, San Diego, CA). The clamp electrodes consist of 2 stainless steel parallel plate calipers (1.5 cm^2) that are placed in contact with the skin so that the leg is held in a semi-extended position throughout pulse administration. The separation distance of the electrodes is described. Typically the leg of the mouse was positioned between the two plates, which were compressed together until snug with a 3-4 mm separation distance between the plates. Two 25 ms pulses at a voltage of 375 V/cm were then generated with a T-820 Electro Square Porator (Genetronics, San Diego, CA). The pulses were administered at a rate of ~1/second.

Dogs were anesthetized with isofluorane for the injection and electroporation procedures. A 6-needle array electrode was used (Genetronics, San Diego, CA) (Jaroszeski, M.J., et al. (1997) *Biochim Biophys Acta* 1334:15-18). The electroporation regimen was 6 pulses of 60 ms duration at a voltage of 200 V/cm. The polarity of the pulse was reversed following each pulse under the control of an Auto Switcher (Genetronics, San Diego, CA). Following the electroporation procedure the skin above injected muscle was tattooed to identify the injection site for later analysis. Carbon particles were also injected in some of the muscles following electroporation as a marker of the injection site for histological analyses.

In one embodiment, the gene delivery approach uses a low voltage (375 V/cm), long pulse (25 ms) electroporation regimen in mice, in contrast to other protocols that use high voltage

(1,800 V/cm) and short pulse (100 μs) parameters (Vicat, J.M., et al (2000) *Hum Gene Ther* 11:909-916).

Serum Assays

Blood samples were collected at the appropriate time points following plasmid administration. Mice were anesthetized IP with Ketamine (60 mg/kg) (Phoenix Scientifics, Inc., St Louis, MO). A proparacaine hydrochloride opthalmic solution (Solvay Animal Health Inc., Mendota Heights, MN) was applied to the eye. The blood was collected in Microtainer® serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) and allowed to clot for 15-30 minutes before centrifuging at 7,000 rpm for 5 minutes. Serum levels of SEAP were determined using a chemiluminescence assay (Tropix, Bedford, MA) following the manufacturers instructions.

For F.IX assays, blood samples were obtained from the retro-orbital plexus of mice. Approximately 250 microliters of blood were collected in EDTA microtainer tubes (Becton Dickinson, Franklin Lakes, NJ). The blood was centrifuged at ~5,000g for 5 minutes. Plasma samples were frozen at -80°C and stored until used for analysis. Plasma hF.IX levels were determined using the Asserachrom IX:Ag human F.IX ELISA kit (Diagnostica Stago, France). Purified human F.IX (Sigma, St. Louis, MO) was used to generate a standard curve. For dogs, blood was collected from the jugular vein of conscious animals into EDTA plasma tubes. Reference plasma for the ELISAs was obtained from each animal prior to treatment. Serum levels of erythropoietin were determined using a commercially available ELISA kit from R&D Systems (Minneapolis, MN).

Western Blot Analysis

Purified hF.IX (Sigma, St. Louis, MO) in sample buffer (0.5 M Tris, 1.5% SDS, 4% β-mercaptoethanol, 10% glycerol, 0.03% bromphenol blue) was loaded on a 10% glycine Tris polyacrylamide gel (Novex, San Diego, CA). Following electrophoresis, protein was transferred to a nitrocellulose membrane (Novex, San Diego, CA). The membranes were then incubated first in canine plasma (1:50) from either treated animals or normal dogs (negative control). For the positive control the membrane was incubated in normal canine plasma spiked with rabbit anti-hF.IX antibody (1:1,000 final). The second antibody was either horseradish peroxidase (HRP)-conjugated rabbit anti-canine antibody (Sigma, St. Louis, MO) or HRP conjugated sheep anti-rabbit antibody (Sigma, St. Louis, MO). Bands on the blots were visualized using a peroxidase substrate kit (Vector Laboratories Inc., Burlingame, CA).

Creatine Kinase (CK)

Serum collected from the dogs was frozen and shipped on dry ice by overnight courier to IDEXX Veterinary Services (West Sacramento, CA) for analysis of CK levels by standard methodology.

Histological Analysis and Fiber-Typing

For hF.IX immunohistochemistry in mouse tissue a method modified from Herzog et al. (1997) *Proc. Natl. Acad. Sci. U S A* 94(11), 5804-5809, was used. Briefly, 10 micrometer cryosections of tissue were fixed in 3% paraformaldehyde for 15 minutes, rinsed in PBS, treated with methanol for 10 minutes, washed three times in PBS and then blocked in 20% normal goat serum. Sections were subsequently incubated for

1 hour with an affinity-purified rabbit anti-hF.IX (Dako Corp., Carpinteria, CA.) that was diluted 1:6,000 in PBS/1%BSA. The sections were rinsed PBS and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA.) diluted 1:400 in PBS for 30 minutes. The sections were rinsed and hF.IX staining was visualized using the Elite ABC reagent (Vector Laboratories, Burlingame, CA.) at a dilution of 1:80 for 30 minutes followed by a 5 minute incubation in a DAB solution (Vector Laboratories, Burlingame, CA.). The sections were counterstained with Mayer's hematoxylin (VWR, Houston, TX). All incubation steps were at room temperature.

For ATPase fiber subtyping, 10 micrometers of muscle tissue cryosections (serial sections of those used for the hF.IX staining) were incubated for 5 minutes in barbital acetate buffer, pH 4.6, transferred to ATPase solution, pH 9.4, for 20 minutes, washed three times in 1% calcium chloride, washed for 5 minutes in 2% cobalt chloride, washed ten times in 0.01 M sodium barbital wash solution, and rinsed in distilled water for 5 minutes. To visualize the ATPase activity, sections were dipped into 1.5% ammonium sulfide for 20 seconds, rinsed in distilled water, dehydrated in ethanol, and coverslipped. At pH 4.6, type I fibers stain dark brown, type IIA fibers stain very light brown and type IIB fibers are intermediate.

For dogs, muscle samples were harvested and immediately placed in 10% neutral buffered formalin overnight at room temperature. The tissue was dehydrated using alcohol and then embedded in paraffin. Sections were cut and stained with Mayer's hematoxylin and eosin (Sigma, St. Louis, MO).

All microscopy was performed with an Olympus BX-40 (Olympus America, Melville, NY) microscope equipped with a DXC-960MD color video camera (Sony Corp., Japan).

EXAMPLE I: Determination of Formulation and Delivery Parameters Using Reporter Genes

Formulating DNA with anionic polymers increases electroporation-mediated gene expression after an intramuscular injection. An example of an anionic polymer is an excess of non-coding DNA, which can increase transgene expression. The protocol that was regularly used to transfect the myofibers of CD-1 or C57BL/6 mice consisted of an injection of a DNA solution followed, two minutes later, by the electroporation of the injected muscle with a clamp electrode. A constant mass (0.75 micrograms, 2.5 micrograms or 15 micrograms) of a plasmid DNA coding for the SEAP (human placental secreted alkaline phosphatase) gene with various amounts of an empty plasmid was co-injected in the tibialis cranialis muscle of CD-1 mice. Empty plasmid means that the plasmid does not carry the coding sequences for SEAP or, preferably, any other gene.

Figure 1 shows SEAP serum concentrations at day 7 post injection of SEAP pDNA/empty DNA mixtures in the tibialis cranialis muscle of CD-1 mice and electroporation of the tissue. Various SEAP pDNA amounts (0.15 micrograms, 0.75 micrograms, 2.5 micrograms, 6.25 micrograms and 15 micrograms) and empty pDNA excess (relative to the coding pDNA) were administered in 50 microliters per animal (half this dose per leg). For each dose of SEAP pDNA tested, SEAP concentration in the serum at the peak of expression (day 7 post

injection/electroporation) increased substantially when a 2-fold excess of empty pDNA was co-administered with the coding pDNA. For instance, SEAP expression in these conditions with 2.5 micrograms SEAP pDNA was similar to that obtained with 6.25 micrograms SEAP pDNA without an empty plasmid. When the amount of SEAP pDNA administered was 2.5 or 15 micrograms, increasing further the excess of empty vector (6, 30 and 120-fold) resulted in a continuous decrease of SEAP expression. Conversely, for the lowest amount of coding pDNA (0.75 micrograms), SEAP expression was maintained when a 6-fold excess of empty DNA was co-injected.

This non-monotonous evolution of SEAP expression as the amount of empty DNA pre-mixed with the SEAP pDNA is increased reflects the interplay of two phenomena. First, the addition of the empty pDNA enhances gene expression due to the saturation of a DNA degradation mechanism or the saturation of a process that deactivates the DNA (e.g., binding to cationic entities such as divalent cations or histones, in the interstitial fluid and in the myocytes nuclei, respectively). This effect can result either in an increased intracellular (or intranuclear) uptake or in a more efficient processing of the SEAP pDNA in the nucleus. Second, the empty vector competes with the SEAP-coding DNA in some of the steps that leads to transcription of the transgene, which results in a decrease of SEAP expression. These steps include the distribution of the DNA in the interstitial fluid prior to electroporation, the intracellular entry through the electropores, the trafficking to the nuclei, the entry in the nuclei and the binding to transcription factors.

Thus, polynucleotides having non-coding sequences or preferably random sequences may function to protect against degradation *in vivo* of plasmid carrying a gene intended to be expressed in an animal.

In addition to using polynucleotides or empty plasmid to enhance transgene expression and protect against degradation, other anionic polymers may also be used. These anionic polymers may include poly-amino acids (such as poly-L-glutamic acid, poly-D-glutamic acids, poly-L-aspartic acid, poly-D-aspartic, and combination thereof) or poly-organic acids (such as poly-acrylic acid) which exhibit beneficiary effects similar to the empty plasmid, but which do not compete with the SEAP pDNA in the processes described above.

Some anionic polymers were found to be considerably more potent than non-coding DNA to increase transgene expression. Anionic polymers with various origins, molecular weights, conformations and charge densities were mixed at various concentrations with the SEAP pDNA (0.05 mg/ml) prior to injection in the tibialis cranialis muscle of CD-1 mice. Seven days after the injection/electroporation procedure (at the peak of expression), SEAP serum concentrations were determined (Fig. 2). At the low DNA dose tested (1.25 micrograms per tibialis), some of the anionic polymers selected considerably increased SEAP expression. The highest SEAP levels were obtained with the 60 kDa poly-acrylic acid (pAA) at 3.0 mg/ml and the 2-15 kDa poly-L-glutamic acid at 6.0 mg/ml. Co-administration of these anionic polymers with the SEAP pDNA enhanced expression by 10 and 8-fold, respectively (Fig. 2).

In order to characterize further the beneficiary effect provided by the anionic polymers, the same type of experiment

as that mentioned above was carried out, but at a 10-fold higher DNA concentration of 0.5 mg/ml. Figure 2 shows SEAP serum concentrations at day 7 post injection of naked SEAP pDNA or SEAP pDNA/anionic polymer mixtures in the tibialis cranialis muscle of CD-1 mice and electroporation of the tissue. The amount of SEAP pDNA administered per animal was 2.5 micrograms in 50 microliters (half this dose per leg). The concentration of the anionic polymer in the injected solution varied as indicated on the graph. Figure 3 shows the same thing as Fig. 2, except that the amount of SEAP pDNA administered per animal was regularly (unless mentioned) 25 micrograms in 50 microliters (half this dose per leg). The concentration of the anionic polymer (or anionic monomer when applicable) in the injected solution varied as indicated on the graph.

At this high DNA concentration, the range of enhancements in SEAP expression resulting from the addition of an anionic polymer was lower than that observed previously (Fig. 2, 3). In particular, the poly-acrylic acids, highly efficient at a low DNA dose, were almost inactive. However, the polypeptides still increased SEAP expression substantially (up to 2-fold with the 2-15 kDa poly-L-glutamic acid at 6.0 mg/ml). This result was particularly remarkable given that SEAP expression was reaching a plateau at this concentration of DNA. Indeed, when the DNA was administered "naked", SEAP expression was enhanced by only 50% and 15% following an increase in DNA concentration by 3-fold (from 0.5 mg/ml to 1.5 mg/ml) and 10-fold (to 5.0 mg/ml), respectively (Fig. 3).

The fact that the L-glutamic acid monomer was unable to increase expression, in contrast to the 2-15 kDa polymer (Fig. 3), demonstrated that a macromolecule is necessary to provide

the effect that leads to higher expressions. When the results from the two separate experiments partially displayed in Fig. 2 and Fig. 3 are gathered in the composite graph (Fig. 5A), the evolution of SEAP expression as a function of DNA concentration can be compared for the naked DNA injection and two of the DNA/anionic polymers treatments (namely DNA / 2-15 kDa poly-L-glutamic acid at 6.0 mg/ml and DNA / 60 kDa poly-acrylic acid (pAA) at 3.0 mg/ml). Two different trends appear clearly after adding an anionic polymer to the DNA solution. In the case of the 60 kDa poly-acrylic acid, the increase in SEAP expression (compared to naked DNA) is high but restricted to low and intermediate DNA concentrations. In the case of the 2-15 kDa polyL-glutamic acid, the levels of expression are slightly lower in this range of DNA concentrations, but the beneficiary effect is still substantial at high DNA concentrations.

The injection/electroporation procedure was conducted in the gastrocnemius muscle of CD-1 mice, instead of the tibialis cranialis, to determine if the increase in expression provided by some anionic polymers is specific to the muscle used for expression. The anionic polymers selected were those that yielded the highest levels of expression in the studies described above, i.e., the 2-15 kDa and 50-100 kDa poly-L-glutamic acids as well as the 60 kDa poly-acrylic acid. Two DNA concentrations were tested in this study, i.e., 0.3 mg/ml (15 micrograms injected per gastrocnemius) and 1 mg/ml. Figure 4 shows SEAP serum concentrations at day 7 post injection of naked SEAP pDNA or SEAP pDNA/anionic polymer mixtures in the gastrocnemius muscle of CD-1 mice and electroporation of the tissue. The amount of SEAP pDNA administered per animal was either 30 micrograms, 100 micrograms or 300 micrograms in 100

microliters (half this dose per leg). The concentration of the anionic polymer in the injected solution varied as indicated on the graph.

The three polymers yielded a substantial increase in expression at the low DNA dose (Fig. 4). Conversely to what was observed when the injections were performed in the tibialis cranialis muscle, the 60 kDa poly-acrylic acid was most efficient at its lowest concentration of 0.6 mg/ml and was less potent than the poly-L-glutamic acids used at 6.0 or 12.0 mg/ml. In the best conditions tested (50-100 kDa poly-L-glutamic acid at 6.0 mg/ml), SEAP expression was increased by 8-fold over that obtained with naked DNA. At the higher DNA concentration, the trends described above were accentuated. The 60 kDa poly-acrylic acid was either inactive or inhibitory at high concentrations, whereas the poly-L-glutamic acids were still yielding a 2 to 3-fold increase in expression. Again, this result was particularly remarkable, given that the expression levels achieved with the naked DNA treatment were only increased by 10% when the DNA concentration was elevated to 3.0 mg/ml instead of 1.0 mg/ml.

Figure 5A shows SEAP serum concentrations at day 7 as a function of the amount of SEAP pDNA injected in the tibialis cranialis muscle of CD-1 mice. Solutions administered two minutes before electroporation consisted of either naked SEAP pDNA or a mixture of SEAP pDNA and a 60 kDa poly-acrylic acid at 3.0 mg/ml or a mixture of SEAP pDNA and a 2-15 kDa poly-L-glutamic acid at 6.0 mg/ml. Figure 5B shows SEAP serum concentrations at day 7 as a function of the amount of SEAP pDNA injected in the gastrocnemius muscle of CD-1 mice. Solutions administered two minutes before electroporation

consisted of either naked SEAP pDNA or a mixture of SEAP pDNA and a poly-L-glutamic acid at 6.0 mg/ml. When the SEAP serum concentration at day 7 post-injection is plotted as a function of the amount of DNA injected per animal as in Figure 5B, the beneficiary effect of the poly-L-glutamic acids (at 6.0 mg/ml) on expression appears clearly.-

EXAMPLE II: Determination of Reporter Gene Expression Using Poly-Glutamic Acid without Electroporation

In order to determine the ability of sodium poly-glutamate to increase the expression of genes encoded on plasmid DNA without electroporation, plasmid DNA formulated in saline was compared with a formulation in sodium poly-glutamate for expression after direct intramyocardial injection in mice.

Plasmid DNA encoding luciferase (pLC0888) was formulated in saline or 6% sodium poly-L-glutamate ((Sigma P4636) at plasmid concentrations of 1 and 3 mg/mL. A total of twenty CD-1 male mice (29-31g) were used. The myocardium was injected directly after surgical exposure. Ten (10) microliters of formulation (using a 3/10 cc insulin syringe) were injected into the apex of the heart (i.e., left ventricle). The heart was repositioned and the thorax sutured. Seven days after injection, the hearts were removed and snap frozen in liquid nitrogen, and stored at -80°C until needed for analysis. For analysis, heart muscle was bead-beat for 2 minutes prior to addition of 1 milliliter of 0.5x Lysis buffer. The tissue was bead-beat for 5 minutes and centrifuged for 10 mins at 13,000 rpm. The supernatants were assayed for luciferase activity. The results of luciferase expression at 7 days after injection are shown in Figure 6. Each bar

represents n = 5. As shown in Figure 6, plasmid DNA formulated with poly-L-glutamate increased gene expression several fold over saline.

EXAMPLE III: Expression of Therapeutic Genes Factor IX Expression Using Polymer Formulations

In addition to reporter genes, experiments were also performed using poly-L-glutamic acids to increase the expression of a therapeutic gene, namely that coding for the coagulation factor IX. The potency of these anionic polymers was tested with pFN0945 (SEQ. ID. NO. 3 and Figure 17) at DNA concentrations (0.5 mg/ml and 1.0 mg/ml) for which hF.IX expression had reached a plateau. Figure 7 shows hF.IX serum concentrations at day 7 post injection of naked hF.IX pDNA or hF.IX pDNA/poly-L-glutamic acid mixtures in the tibialis muscle of C57BL/6 mice and electroporation of the tissue. The amount of hF.IX pDNA administered per animal was either 25 μg (0.5 mg/ml) or 50 micrograms (1.0 mg/ml) in 100 microliters (half this dose per leg). The concentration of the anionic polymer in the injected solution varied as indicated on the graph. The poly-L-glutamic acids selected differed by their molecular weight, ranging from 0.5-1.5 kDa (with a degree of polymerization (DP) of 9) to 15-50 kDa. All poly-L-glutamic acids tested were able to increase hF.IX expression substantially, especially at 6.0 mg/ml, with only small differences in potency between polymers. The highest hF.IX level obtained after injection in the tibialis muscle of C57BL/6 mice and electroporation of the tissue was 280 ng/ml, with a treatment consisting of DNA at 0.5 mg/ml and the 2-15 kDa poly(L-glutamic acid) at 6.0 mg/ml. In comparison, the

naked DNA treatment only resulted in hF.IX levels around 160 ng/ml.

Persistence of Expression from Plasmid DNA

To determine if hF.IX expression could persist in the plasma for an extended time in the absence of an immune response, plasmid formulated with PVP (5%) was tested in immune deficient SCID beige mice. Figure 8 shows hF.IX expression in plasma of immune deficient (SCID beige) mice. Mice were initially injected with plasmid (1 mg/ml) formulated with 5% PVP (25 microliters each tibialis muscle and 50 microliters in each gastrocnemius muscle). Consistent with expression patterns in immune competent mice, hF.IX levels peaked 7 days after injection at ~120 ng/ml (Figure 8). Following a 35% drop in hF.IX levels by 14 days after injection, expression remained fairly stable to 90 days post injection but had fallen to ~20% of peak values by day 125.

At day 153, the animals were re-injected with plasmid and electroporated in the same muscles that were used in the first treatment. For the second injection at day 153 (indicated by the arrow), the animals were separated into two groups. One group was injected with plasmid formulated with 5% PVP (n = 7) and the other group injected with plasmid formulated with 6 mg/ml poly-L-glutamate (n = 8). The second injections utilized the same injection sites and plasmid dose that were used for the first injections. In both groups of SCID mice, plasmid re-administration led to a significant rise in plasma hF.IX levels. The group injected with plasmid formulated with poly-L- glutamate had significantly higher expression than the group injected with PVP. This difference in expression levels

between the groups following the second administration was maintained throughout the duration of the experiment. The kinetics of hF.IX expression in both groups were similar to that seen after the first administration in that there was a significant drop from peak expression (obtained ~ 7 days after re-injection) within the first two weeks.

The graphs in the insert of Figure 8 also show the effect of 6 mg/ml poly-L-glutamate on hF.IX and hEPO expression in comparison to saline. For these experiments, the tibialis of mice were injected with plasmid coding for hF.IX (50 micrograms) or for human erythropoietin (75 micrograms) followed by electroporation. Plasma or serum samples were collected 7 days after treatment for analysis. All values are represented as mean ± SEM. A Students t-test was used to compare means and in Figure 8, * = $P \leq 0.05$. Plasmids formulated with poly-L-glutamate (6 mg/ml) led to a 1.5 fold to 5.9 fold enhancement in expression compared to plasmid in saline with electroporation and was dependent on the inserted gene (Figure 8, insert).

In the SCID mice at 10 months after the initial injection with PVP followed by reinjection with a poly-L-glutamate formulation, the tibialis and gastrocnemius muscles were harvested for hF.IX immunostaining and muscle fiber typing. Figure 9 shows immunohistology and fiber-type of hF.IX expressing myocytes in SCID mouse muscle. Representative sections of SCID mouse gastrocnemius muscle from tissue that was harvested ~300 days after the initial injection. Figure 9A shows hF.IX immunolocalization wherein positive myocytes are stained dark (original magnification 100X). Figure 9B shows ATPase staining (pH 4.6) of a serial section of panel A. Type

I fibers (dark) and type II fibers (light) are distinguished (original magnification 100X). A representative sample of complementary fibers are labeled in both panels indicating both type I and type II fibers are expressing hF.IX. Both the tibialis and gastrocnemius muscles showed a broad distribution of fibers expressing hF.IX. In the gastrocnemius, expression was found in both type I and type II fibers in roughly equal proportions although the absolute number of stained type I fibers was much lower than type II fibers (Figure 9). In the mouse tibialis there were few if any type I fibers and thus expression was observed primarily in type II fibers. Thus, long-term expression of hF.IX, achieved in immune compromised (SCID beige) mice, indicates that plasmids are stable and transcriptionally active in muscle for a prolonged period of time.

Applicability to Large Animals

The applicability of the gene delivery procedure to large animals is a necessary prerequisite step for the development of a potentially clinically useful gene therapy. Figure 10A depicts the results of plasma hF.IX levels in dogs following intramuscular injection of plasmid augmented by electroporation. Six adult dogs (beagles 9-13 kg) were injected with ~1.6 or ~2.8 mg/kg of plasmid using a multiple site protocol and followed by electroporation with 6-needle array electrodes. The DNA was formulated with poly-L-glutamate (6 mg/ml) for these studies. The dogs were divided into two groups. In one group a total dose of 18 mg was administered intramuscularly divided into 6 sites, one in each of the biceps femoris, semimembranosus and cranial tibialis

muscles of both rear legs. In the second group, 36 mg of plasmid was administered intramuscularly into 12 sites, one each in the biceps femoris, semimembranosus, semitendinosus, vastus lateralis, cranial tibialis and long head of the triceps brachii muscles of the front and rear limbs. A total volume of 2.0 ml was administered to each site. At each site 2.0 ml of plasmid (1.5 mg/ml) formulated with 6.0 mg/ml poly-L-glutamate was injected followed by electroporation with a 6-needle array electrode. The 6 and 12 injection site groups had 18 mg and 36 mg of plasmid injected per animal, respectively. Figure 10A shows the results where plasma was collected and analyzed by ELISA. Values are means ± SEM with n = 3 for each group.

Mean values of the 12 and 6 injection site groups peak at 36.1 ng/ml (day 22) and 27.2 ng/ml (day 14), respectively (Figure 10A). The values for the two groups diverged at day 22 due to an unexpected increase in mean expression in the group of animals injected at 12 sites. However, the expression levels in this group at day 22 are not significantly higher than at day 14. Regardless of this anomaly, by day 28 expression levels of both groups were indistinguishable from background levels.

Immune Response to Expressed Protein

Figure 10B shows a western blot of purified hF.IX using treated animal serum as the primary antibody. Lane A represents the molecular weight marker; lane B represents the negative control (i.e., serum from untreated animals); lane C represents the positive control (i.e., canine serum spiked with rabbit anti-hF.IX antibodies); lane D represents the

immunoreaction to hF.IX by the serum from a female dog from the 6 injection group (peak expression hF.IX 35.71 ng/ml); lane E represents the immunoreaction to hF.IX by the serum from a male dog from the 12 injection group (peak hF.IX expression 47.9 ng/ml). Thus, analysis by Western blot indicated that plasma from the dogs contained material that cross-reacted with purified hF.IX consistent with an immune response to the human protein (Figure 10B).

Furthermore, serum analysis also revealed a transient increase in creatine kinase (CK) levels that peaked two days after treatment, and returned to normal levels by 7 days after treatment indicating some muscle trauma is associated with the gene delivery procedure using invasive 6-needle array electrodes. This response is clearly dose dependent with the animals administered the higher dose (12 injection sites) having higher peak levels of CK on day 3 than did the animals from the 6 injection sites group. A histological examination of the different injected muscles revealed some muscle damage approximately 1 month after treatment. In most instances, no histological changes were noted or were restricted to small focal points, where there were indications of myocyte loss and infiltrating monocytes. In rare instances, the injection site was characterized by areas of necrotic tissue and associated myocyte loss. This type of damage was also observed in mice at earlier time points after treatment (2 weeks) when the caliper electrodes were used, but the muscles recovered to normal histology over time (data not shown). There was no indication that a particular muscle type was more susceptible to tissue damage than another.

Expression is Dose Dependant

To establish that expression of hF.IX in canine muscle was dose-dependent, biceps femoris and tibialis cranialis of the left and right hindlimbs of 11-week-old dogs were used for the gene delivery protocol. Formulated plasmid was injected into 4 sites in each dog (left and right tibialis cranialis, left and right biceps femoris). The plasmid concentration was 3.0 mg/ml. Injected volumes (at each site) were 0.12 ml, 0.36 ml, 0.60 ml and 1.2 ml for each group. Serum was collected 7 days after treatment for analysis (peak levels). To normalize for variations in the animals' weight, absolute hF.IX levels are represented (determined by estimating blood volume at 7% of the dogs weight). Values are means ± SEM with n = 3 for each group. Values are means ± SEM per animal with n = 4 for each group. Plasma hF.IX levels increased with increasing amounts of plasmid from 0.8 mg/kg up to 2.3 mg/kg. At high doses of plasmid (5.3 mg/kg) mean expression levels were lower than obtained at the 2.3 mg/kg dose but the difference was not significant.

Using plasmid injected into skeletal muscle followed immediately with electroporation, we have achieved therapeutically significant levels of hF.IX expression in the plasma of mice and dogs.

Optimized hF.IX Sequence

The above experiments were performed with plasmid pFN0945 (SEQ. ID. NO. 3 and Figure 17), which has the natural human nucleic acid sequence encoding for hF.IX. For gene therapy applications in human, pFN0945 may also be used, but a codon optimized sequence for hF.IX may be preferred when higher

expression is desired due to higher translation of a codon optimized mRNA. An example of a codon optimized sequence for hF.IX is plasmid pFN1645, which is disclosed as SEQ. ID. NO. 4 and shown in Figure 18.

EXAMPLE IV: Expression of Therapeutic Genes

The ability of poly-L-glutamate to increase the expression of a non-viral erythropoietin ("EPO") gene was also undertaken. Using quantitative polymerase chain reaction (qPCR) analysis, plasmid formulated in Poly-L-Glutamate resulted in at least a log increased levels of mEPO DNA compared with animals receiving a saline/DNA formulation.

EPO Expression Using Polymer Formulations

The mEPO coding sequence was inserted into the Valentis plasmid backbone containing a 107 bp 5'UTR, a 117 bp synthetic intron, the human growth hormone polyadenylation signal, a PUC12 origin of replication and a kanamycin resistance gene as aforementioned. The mEPO gene was driven by the CMV enhancer/promoter. The complete sequence of the resulting plasmid pEP1403 containing the mEPO gene is disclosed in the sequence listing as SEQ. ID. NO. 2 and the plasmid map is shown in Figure 19. Plasmids were grown in *Escherichia coli* DH5α and were purified using a proprietary method involving alkaline lysis and chromographic methods (Abruzzese, R.V., et al. (1999) *Hum Gene Ther* 10:1499-1507, incorporated herein by reference).

Animals received CMV-mEPO formulated either in 15-50 kDa poly-L-glutamate or in saline. Plasmid formulations were injected intramuscularly in each leg, 25 microliters in each tibialis, 50 microliters in each gastrocnemius followed by

electroporation 2 min after injection (375 V/cm (113 V/0.3 cm), 2 pulses, 25 msec pulse length. At defined time intervals, blood was collected by retro-orbital methods and hematocrit levels determined or the serum assayed for EPO levels.

At indicated times, total muscle DNA was extracted and levels of were quantified by qPCR as follows: Plasmid DNA quantities in mouse muscles were determined by conducting TaqMan real time quantitative PCR (Applied Biosystems, Foster City, CA) on isolated DNA samples as previously described (Mahato, R.I. *et al. Hum. Gene Ther.* 9, 2083-2099 (1998)). The primers used in the PCR were a forward primer, which primes in the 5' untranslated region, and a reverse primer, which primes in the mouse EPO coding region. The probe sequence was located within the EPO gene. Purified CMV-mEPO plasmid DNA was used to generate a standard curve for the PCR assay. As shown in Figure 11, formulation in poly-L-glutamate results in a several fold increase in the amount of plasmid DNA that can be detected in tissues after electroporation.

For mEPO expression determination, 75 mg pEP1403 (SEQ. ID. NO. 2) in 150 ml was delivered to C57BL/6 mice, 25 microliters per tibialis, 50 microliters per gastrocnemius. Plasmid was formulated in saline or 6 mg/mL poly-L-glutamate. Figures 12 and 13 depict mEPO expression and Figure 12 also depicts the hemotocrit level in mice following delivery of the mouse EPO gene by electroporation using saline and sodium poly-L-glutamate formulations.

As shown in Figures 12 and 13, delivery in a poly-glutamate formulation results in considerably higher levels of expressed protein than when the plasmid DNA is delivered in saline. Because a very small amount of erythropoietin is

required to give a maximal increase in hematocrit, the induced hematocrit levels shown on Figure 12 do not differ between saline and polyglutamate formulations. However, because polyglutamate results in more efficient transfection, it is expected that lower amounts of DNA can be administered using polyglutamate formulations.

EXAMPLE V: Expression of Therapeutic Genes

Interferon Alpha Expression Using Polymer Formulations

The hINFα 2b coding sequence was inserted into the Valentis plasmid backbone containing a 107 bp 5'UTR, a 117 bp synthetic intron, the human growth hormone polyadenylation signal, a PUC12 origin of replication and a kanamycin resistance gene. The hINFα gene was driven by the CMV enhancer/promoter. The complete sequence of the resulting plasmid pIF0921 containing the hINF-α gene is disclosed in the sequence listing as SEQ. ID. NO. 1 and the plasmid map is shown in Figure 20. Plasmids were grown in *Escherichia coli* DH5α and were purified using a proprietary method involving alkaline lysis and chromographic methods (Abruzzese, R.V., et al. (1999) *Hum Gene Ther* 10:1499-1507, incorporated herein by reference).

For expression analysis, 25 microliters plasmid formulations either in poly-glutamate or in saline that had varying DNA concentrations (1.0 mg/ml, 0.1 mg/ml and 0.01 mg/ml) were injected into each tibialis-both legs were electroporated with caliper electrodes at 375V/cm, 2 pulses, 25 ms each pulse. For analysis, serum was collected via retro orbital bleeds (days 4, 7, 14 and 30). A commercially available ELISA (Endogen) was used to determine INF-α levels. As shown in Figures 14A and B, a significant enhancement of

hINF-α expression in CD-1 mice was obtained using plasmid formulated with 6 mg/ml poly-L-glutamate at both 5 and 50 microgram DNA doses.

EXAMPLE VI: Nuclease Protection of Plasmid DNA formulated in Poly-L-Glutamate

Experiments were undertaken to determine the ability of poly-L-glutamate and Pluronic F68 to protect plasmid DNA from nuclease digestion. DNase I was obtained from Gibco/BRL (#18068-015). The sodium salt of poly-L-glutamic acid, 2 - 15kDa was obtained from Sigma. Pluronic F68 was obtained from Spectrum. Polymer/DNA 2x stock solutions were prepared (Pluronic F68 = 200 micrograms/ml plasmid DNA in 10% F68; Poly-L-glutamate = 200 micrograms/ml plasmid DNA in 12 mg/ml sodium poly-L-glutamate). DNase dilutions from 1:10 to 1:10,000 were prepared in 1x DNase buffer. The final reaction mixtures included 25 microliters of the formulation, 15 microliters of water, 5 microliters of 10x DNase buffer and 5 microliters of Dnase that were added in the order listed. The reaction mixtures were incubated for 15 minutes at 37° C. and terminated by addition of EDTA prior to gel electrophoresis.

The results of the DNase protection assay are shown in Figure 15. Panel A represents a DNA in saline formulation; Panel B represents DNA formulated in 5% Pluronic F68; Panel C represents DNA formulated in 6 mg/ml poly-L-glutamate. Lane A represents the negative control (i.e., plasmid DNA without Dnase); lane B represents the positive control (i.e., plasmid DNA and DNase mixed 1:1); lanes C-G represents the experimental conditions wherein DNA formulated with either saline (Panel A), F68 (Panel B), or poly-glutamate (Panel C) were mixed with

DNase diluted 1:1 (lane C); 1:10 (lane D);1:100 (lane E); 1:1,000 (lane F); and 1: 10,000 (lane G). In saline, DNase at 1:100 is able to abolish the lower band of supercoiled plasmid in addition to degradation of the DNA resulting in a smear of different molecular weights on the gel. In contrast, both poly-L-glutamate and Pluronic F68 were able to confer protection from DNase degradation at 1:100 dilution.

EXAMPLE VII: Long-Term Biological Stability of DNA formulated in Poly-L-Glutamate

Experiments were also undertaken to evaluate the stability of liquid poly-L-Glutamate (15-50 kDa)/DNA formulations.

Animals:

108 CD-1 mice (29-31g) were obtained from Charles Rivers Labs. The animals were housed in microisolators (10 mice per isolator) in the Laboratory Animal Resource (LAR) vivarium and maintained at 12 /12 h day/night cycle, room temperature 72° F (23° C), and humidity 40%. Food (Purina rodent chow) and water was provided *ad libitum*. Combination anesthesia consisting of a mixture of Ketamine (74.0 mg/ml), Xylazine (3.7 mg/ml), and Acepromazine (0.73 mg/ml) was administered IP at a dosage of 1.8-2.0 ml/kg.

Treatment Groups and Routes of Administration:

The animals were randomly divided into treatment groups with 6 (tibialis) or 5 (gastrocnemius) mice/group. For the tibialis groups, 25 microliters of the formulations described below were injected in each tibialis muscle, i.e. 50 microliters in total volume per mouse. For the gastrocnemius

groups, 50 microliters of the formulations described below were injected in each gastrocnemius muscle, i.e. 100 microliters in total volume per mouse.

Formulations

Formulations were prepared in 150 mM NaCl, 5 mM Tris-HCl, pH 7.5. SEAP encoding plasmid pAP1166.157 at 1 mg/ml was used. Plasmid and poly-L-Glutamate (15-50 kDa) were formulated as follows.

Formulation	pDNA conc. (mg/ml)	salt	Poly-L-Glu	Buffer
A	1.0	150 mM	6.0 mg/ml	5 mM Tris/pH 7.5
B	0	150 mM	6.0 mg/ml	5 mM Tris/pH 7.5

For the liquid formulations, A (0.5 ml) and B (1.5 ml) of the same storage conditions were mixed (or rehydrated with water and mixed for the lyophilized samples) right before use for *in-vivo* testing (in the gastrocnemius and tibialis muscles of CD-1 mice) and QC analysis. The final DNA concentration of the mixture was 0.25 mg/ml. Each A_n/B_n couple was tested at day 8, 21, 60 and 105. As a control, a fresh sample of 0.5 ml of A and 1.5 ml of B was tested at every time point. As a fresh naked DNA control, a sample of 0.5 ml of A (A not including poly-L-Glutamate) and 1.5 ml of B (B not including poly-L-Glutamate) was tested at every time point.

The lyophilization/storage conditions for which results are shown in Figure 16 were the following:

Group	Physical storage condition	Temperature
A	Lyophilization (storage N.A. for the sample tested right after completion of the lyophilization cycle)	+4°C
B	Liquid	-20°C
C	Liquid	+4°C
D	Liquid	+25°C
E	Liquid	+37°C
F	Liquid	+50°C
G	Liquid/storage with a freeze/thaw/ freeze cycle at day 2, 4 (and 10, 17, 24, 31, 38, 45, 52 and 59 if applicable)	-20 °C
H	Fresh DNA/pGlu	
I	Fresh naked DNA	

Figure 16 depicts the results of the final 105 day time point and indicates the biological activity of the DNA under different storage conditions. As indicated on Figure 16, plasmid DNA at 1 mg/ml formulated in poly-L-glutamate at 6 mg/ml is stable for over three months in liquid solution at room temperature. Poly-L-glutamate also protected the DNA against degradation during freeze thawing and lyophilization.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur

to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

Those references not previously incorporated herein by reference, including both patent and non-patent references, are

expressly incorporated herein by reference for all purposes. Other embodiments are within the following claims.

Claims

1. A formulation for delivery of a nucleic acid molecule to a cell, comprising a nucleic acid and an anionic polymer, wherein the anionic polymer is non-encapsulating.

2. The formulation of claim 1, wherein the formulation is non-condensing.

3. The formulation of claim 1, wherein said anionic polymer is selected from the group consisting of: poly-nucleic acids, poly-amino acids, poly-acrylic acid, poly galacturonic acid, and poly vinyl sulfate.

4. The formulation of claim 3, wherein the anionic amino acid polymer is selected from a group consisting of: poly glutamic acid, poly aspartic acid, a polymer consisting of glutamic acid and aspartic acids, and salts thereof.

5. The formulation of claim 4, wherein the poly-L-glutamic acid is characterized by a molecular weight in the range from 2,000 to 100,000 Daltons.

6. The formulation of claim 5, wherein the poly-L-glutamic acid or poly-aspartic acid is characterized by a molecular weight in the range from about 15,000 to about 50,000 Daltons.

7. The formulation of claim 5, wherein the poly-L-glutamic acid or poly-aspartic acid is characterized by a molecular weight in the range from about 2,000 to about 15,000 Daltons.

8. The formulation of claim 5, wherein the poly-L-glutamic acid or poly-aspartic acid is characterized by a molecular weight in the range from about 50,000 to about 100,000 Daltons.

9. The formulation of claim 3, wherein the poly-amino acid or salt thereof is formulated with the nucleic acid molecule at a polymer concentration ranging from 1 to 12 mg/ml.

10. The formulation of claim 9, wherein the poly-amino acid or salt thereof is formulated with the nucleic acid molecule at a polymer concentration of about 2 to about 6 mg/ml.

11. The formulation of claim 1, wherein the formulation is isotonic.

12. The formulation of claim 1, wherein the polymer enhances delivery of the nucleic acid to the cell *in vivo*.

13. The formulation of claim 12, wherein the polymer enhances delivery of the nucleic acid to a cell in a muscle tissue *in vivo*.

14. The formulation of claim 12, wherein the polymer enhances delivery of the nucleic acid to multiple cell lines *in vivo*.

15. The formulation of claim 1, wherein the polymer confers stability to the nucleic acid during storage conditions selected from the group consisting of: liquid storage, lyophilization and freezing.

16. The formulation of claim 1, wherein the nucleic acid molecule comprises a sequence encoding a protein selected from the group consisting of growth hormones, growth factors, cytokines, clotting factors, antigens, antigenic and anti-antigenic factors.

17. The formulation of claim 16, wherein the clotting factor is a Factor IX.

18. The formulation of claim 16, wherein the growth factor is an erythropoietin.

19. The formulation of claim 16, wherein the cytokine is an interferon.

20. The formulation of claim 10, further comprising a buffer suitable for internal administration.

21. The formulation of claim 20, comprising the nucleic acid formulated with poly-L-glutamate at a concentration of about 6 mg/ml and about 150mM NaCl.

22. The formulation of claim 21, wherein the nucleic acid is present in the formulation at about 1 mg/ml.

23. The formulations of claim 21 or 22, further comprising a Tris buffer at a concentration ranging from about 5 mM to about 10 mM.

24. A lyophilized nucleic acid formulation comprising a nucleic acid encoding a functional molecule and an anionic polymer, wherein the anionic polymer is non-encapsulating.

25. The formulation of claim 24, wherein the anionic polymer is selected from the group consisting of: poly-nucleic acids, poly-amino acids, poly-acrylic acid, poly galacturonic acid, and poly vinyl sulfate.

26. The formulation of claim 25, wherein the poly-amino acid is selected from a group consisting of: poly glutamic acid, poly aspartic acid, a polymer consisting of glutamic acid and aspartic acids, and salts thereof.

27. The formulation of claim 26, wherein the poly-glutamic acid is a poly-L-glutamic acid.

28. The formulation of claim 27, wherein the salt of poly-L-glutamic acid is a sodium salt and is present in the formulation at a concentration of 1 to 12 mg/ml prior to lyophilization.

29. The formulation of claim 28, wherein the sodium salt of poly-L-glutamic acid is present in the formulation at a concentration of about 6 mg/ml prior to lyophilization.

30. A plasmid DNA composition suitable for internal administration comprising about 1 mg/ml plasmid DNA, about 6 mg/ml poly-L-glutamate, about 150mM NaCl and about 10 mM Tris, pH 7.5.

31. The plasmid DNA composition of claim 30, wherein the composition is stored in a lyophilized state and is reconstituted prior to administration.

32. A method of administering to a mammal a composition for delivery of a nucleic acid molecule to a cell, comprising

the step of introducing the composition of any of claims 1-27 into a tissue of a mammal.

33. The method of claim 32, wherein said step of introducing said composition into a tissue of a mammal is by injection.

34. The method of claim 33, wherein said tissue is muscle.

35. The method of claim 32, wherein said tissue is a tumor.

36. The method of claim 32, further comprising the step of electroporating the tissue.

37. The method of claim 36, wherein the electroporating is performed through the use of a device configured and arranged to cause pulse voltage delivery of said composition.

38. The method of claim 33, wherein said method induces an immune response.

39. A composition for gene delivery *in vivo* comprising an anionic polymer and a nucleic acid encoding a gene product, and wherein the polymer is non-encapsulating.

40. The composition of claim 39 wherein said gene product comprises a therapeutic protein.

41. The composition of claim 39 wherein said gene product comprises an antigen.

42. The composition of claim 39, wherein the anionic polymer is a poly-L-glutamate.

43. The composition of claim 39, wherein the nucleic acid encodes an erythropoietin molecule.

44. The composition of claim 39, wherein the nucleic acid encodes a clotting factor.

45. The composition of claim 39, wherein the nucleic acid encodes a cytokine.

46. The use of an anionic polymer for the preparation of a nucleic acid formulation wherein the formulation is delivered *in vivo* to an animal tissue in conjunction with *in vivo* electroporation of the tissue.

47. The use of claim 46 wherein the anionic polymer is a poly-L-glutamate.

48. The use of claim 47, wherein the poly-L-glutamate is formulated at a concentration of 1 to 12 mg/ml with the nucleic acid.

49. A kit comprising a container for providing a composition of claim 1 and either (*i*) a pulse voltage device for delivering said composition to cells of an organism, wherein said pulse voltage device is capable of being combined with said container, or (*ii*) instructions explaining how to deliver said composition with said pulse voltage device.

50. A method for making a kit of claim 44 comprising the steps of combining a container for providing a composition of

claim 1 with either (*i*) a pulse voltage device for delivering said composition to the cells of an organism, wherein said pulse voltage device is capable of being combined with said container, or (*ii*) instructions explaining how to deliver said composition with said pulse voltage device.

51. A method of treating a mammal suffering from cancer or an infectious disease, comprising the step of providing a composition of claim 1 to cells of said mammal by use of a device configured and arranged to pulse voltage delivery of said composition to cells of said mammal, wherein said molecule encodes a cancer antigen or an antigen for said infectious disease.

52. The method of claim 51, wherein said cancer antigen is MAGE 1, and said cancer is melanoma.

53. The method of claim 51, wherein said infectious disease antigen is HBV core antigen, and said infectious disease is chronic hepatitis.

54. A formulation for delivery of a nucleic acid molecule to a cell, comprising a nucleic acid encoding a gene product and an excess of non-coding DNA.

55. The formulation of claim 54 wherein said gene product comprises a therapeutic protein.

56. The formulation of claim 54 wherein said gene product comprises an antigen.

57. The formulation of claim 54 wherein said non-coding DNA comprises an empty plasmid.

58. The formulation of claim 54 wherein the ratio of nucleic acid encoding a gene product and non-coding DNA is about 1:2 to 1:6.

59. A pharmaceutical composition for increasing a blood level of a therapeutic protein, comprising a non-viral vector encoding the therapeutic protein and a non-cationic compound that enhances transfection.

60. The pharmaceutical composition of claim 59, wherein the non-cationic compound enhances transfection in conjunction with electroporation *in vivo*.

61. The pharmaceutical composition of claim 59 or 60, wherein the non-cationic compound is an anionic amino acid polymer.

62. The pharmaceutical composition of claim 61, wherein the anionic amino acid polymer is a poly-L-glutamate.

63. The pharmaceutical composition of any of claims 59 - 62, wherein the therapeutic protein is selected from the group consisting of: a clotting factor; a growth factor; and a cytokine.

64. The pharmaceutical composition of claim 63, wherein the therapeutic protein is selected from the group consisting of: Factor IX; EPO; and INF-alpha.

65. A stabilized pharmaceutical composition for increasing a blood level of a therapeutic protein, comprising a non-viral vector encoding the therapeutic protein and a non-

cationic compound that protects the vector from biological degradation induced by lyophilization or freezing.

66. The stabilized pharmaceutical composition of claim 65, wherein the non-cationic compound is a poly-glutamate.

67. A method for increasing a blood level of a therapeutic protein in a mammal, comprising the steps of:

a) preparing a formulation comprising a non-viral vector encoding a therapeutic protein and a non-cationic compound;

b) introducing the formulation to a mammalian tissue *in vivo*; and

c) electroporating the tissue, wherein the non-cationic compound increases transfection of the non-viral vector in conjunction with the electroporating of the tissue.

68. The method of claim 67, wherein the tissue is a muscle.

69. The method of claim 67, further comprising the steps of lyophilizing the formulation, storing the lyophilized formulation, and rehydrating the lyophilized formulation prior to introducing the formulation to a mammalian tissue.

70. The method of any of claims 67 - 69, wherein the non-cationic compound is an anionic amino acid polymer.

71. The method of claim 70, wherein the anionic amino acid polymer is a poly-glutamate.

72. The method of any claims 67 - 71, wherein the therapeutic protein is selected from the group consisting of: a clotting factor; a growth factor; and a cytokine.

73. A pharmaceutical composition to be administered to an organism, the pharmaceutical composition comprising:

an anionic polymer, and

a vector comprising a nucleic acid sequence encoding hF.IX.

74. The pharmaceutical composition of claim 73 wherein the nucleic acid sequence comprises codons that are optimized for expressing hF.IX.

75. The pharmaceutical composition of claim 73 wherein the vector further comprises: a 107 bp 5'UTR, a 117 bp synthetic intron, a human growth hormone polyadenylation signal, a PUC12 origin of replication, and a kanamycin resistance gene.

76. The pharmaceutical composition of claim 73 wherein the vector is administered in the range of 0.8mg to 5.3mg per kilogram weight of the organism.

77. The pharmaceutical composition of claim 73 wherein the vector is selected from the group consisting of: SEQ. ID. NO. 3 and SEQ. ID. NO. 4.

78. The pharmaceutical composition of claim 73 wherein the anionic amino acid polymer is poly-glutamic acid or the salt thereof.

79. The pharmaceutical composition of claim 78, wherein the poly-glutamic acid or the salt thereof is characterized by a molecular weight in the range from 2,000 to 100,000 Daltons.

80. The pharmaceutical composition of claim 78, wherein the poly-glutamic acid or the salt thereof is characterized by a molecular weight in the range from about 15,000 to about 50,000 Daltons.

81. The pharmaceutical composition of claim 78, wherein the poly-glutamic acid or the salt thereof is characterized by a molecular weight in the range from about 2,000 to about 15,000 Daltons.

82. The pharmaceutical composition of claim 78, wherein the poly-glutamic acid or the salt thereof is characterized by a molecular weight in the range from about 50,000 to about 100,000 Daltons.

83. The pharmaceutical composition of claim 78, wherein the poly-glutamic acid or salt thereof is formulated with the nucleic acid molecule at a polymer concentration ranging from 1 to 12 mg/ml.

84. The pharmaceutical composition of claim 83, wherein the poly-glutamic acid or salt thereof is formulated with the vector at a concentration of about 2 to about 6 mg/ml of poly-glutamic acid or salt thereof.

85. The pharmaceutical composition of claim 73, wherein the formulation is isotonic.

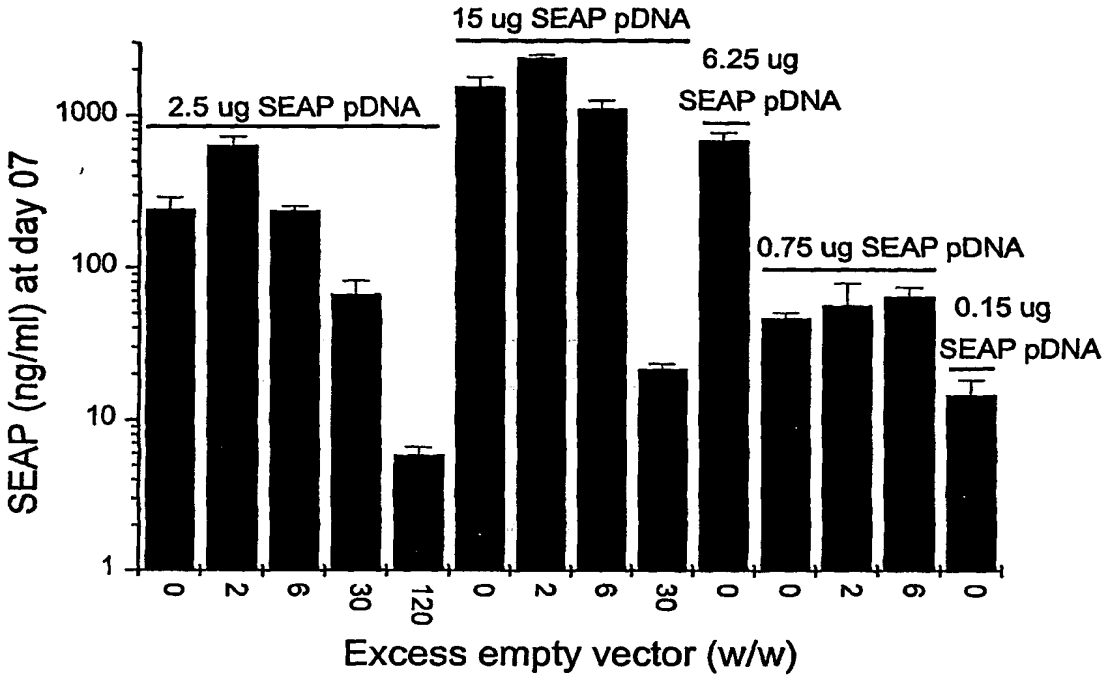

15 ug SEAP pDNA
6.25 ug
SEAP pDNA
2.5 ug SEAP pDNA
0.75 ug SEAP pDNA
0.15 ug
SEAP pDNA
SEAP (ng/ml) at day 07
1000
100
10
1
0 2 6 30 120 0 2 6 30 0 0 2 6 0
Excess empty vector (w/w)

Fig. 1

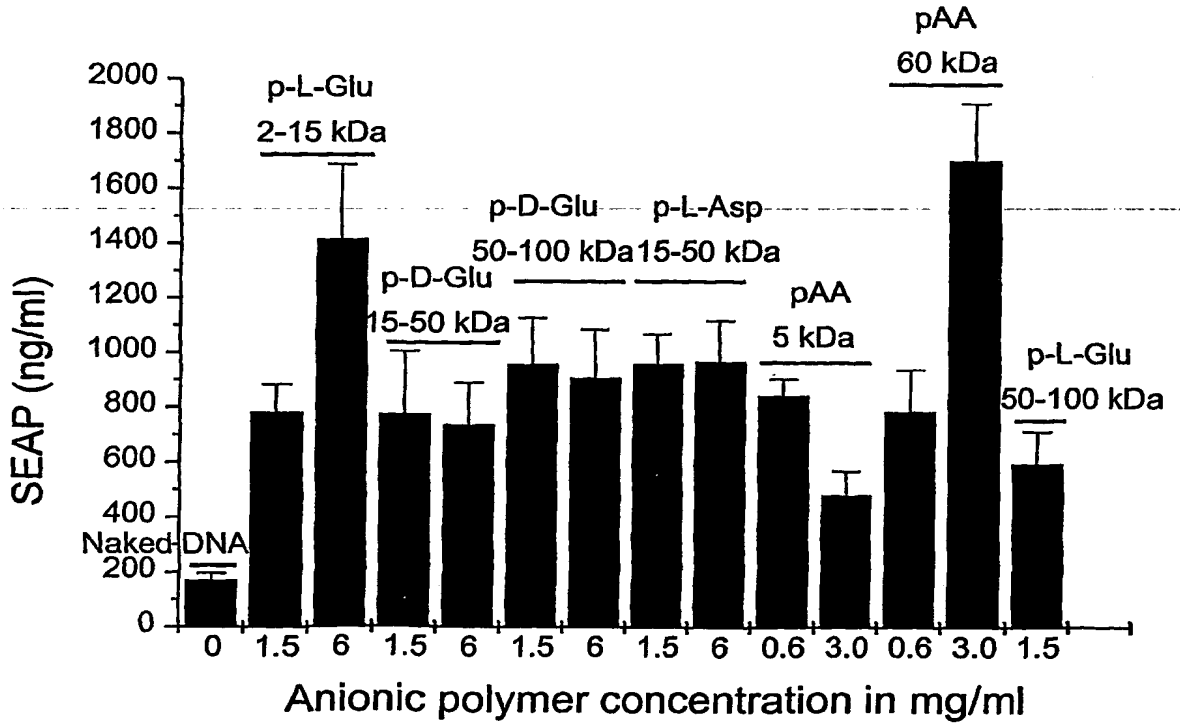

pAA
60 kDa
p-L-Glu
2-15 kDa
p-D-Glu
50-100 kDa
p-L-Asp
15-50 kDa
p-D-Glu
15-50 kDa
pAA
5 kDa
p-L-Glu
50-100 kDa
SEAP (ng/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Naked DNA
0 1.5 6 1.5 6 1.5 6 1.5 6 0.6 3.0 0.6 3.0 1.5
Anionic polymer concentration in mg/ml

Fig. 2

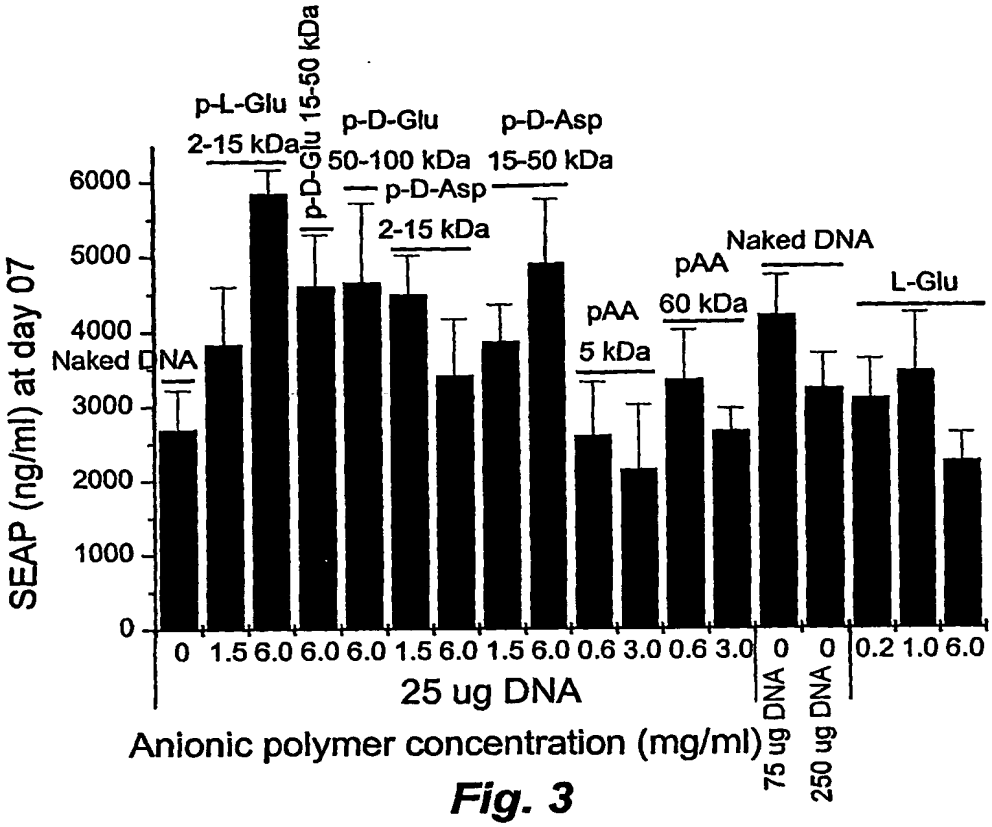

SEAP (ng/ml) at day 07
6000
5000
4000
3000
2000
1000
0
Naked DNA
p-L-Glu 2-15 kDa
p-D-Glu 15-50 kDa
p-D-Glu 50-100 kDa
p-D-Asp 15-50 kDa
p-D-Asp 2-15 kDa
pAA 5 kDa
pAA 60 kDa
Naked DNA
L-Glu
0 1.5 6.0 6.0 6.0 1.5 6.0 1.5 6.0 0.6 3.0 0.6 3.0 0 0 0.2 1.0 6.0
25 ug DNA
75 ug DNA
250 ug DNA
Anionic polymer concentration (mg/ml)

Fig. 3

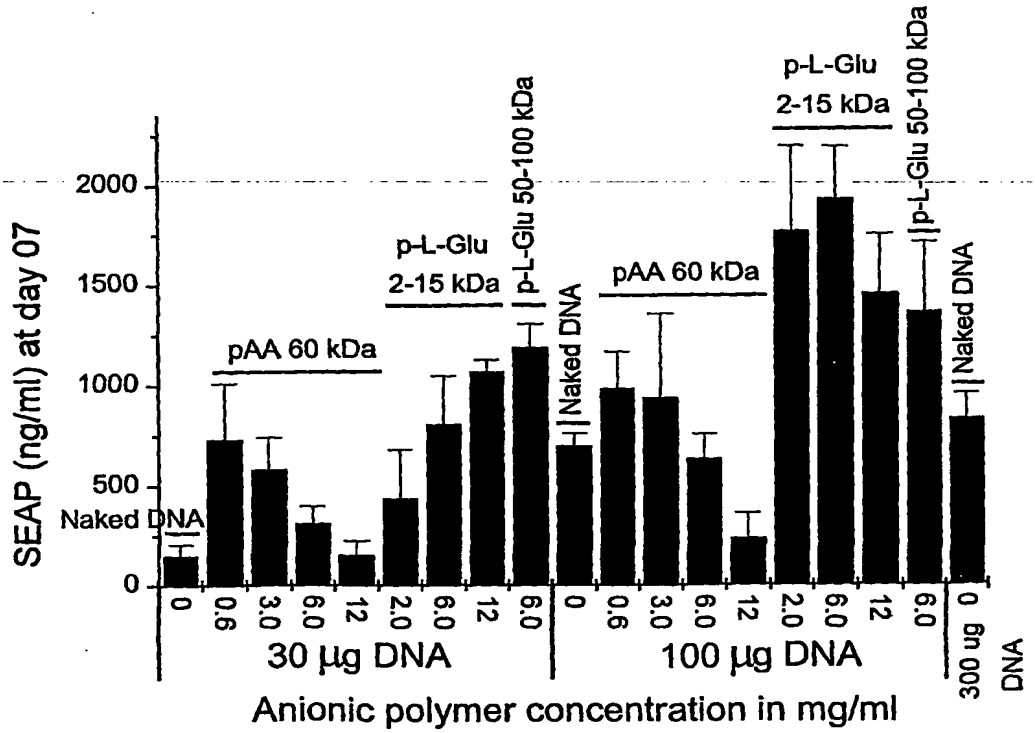

SEAP (ng/ml) at day 07
2000
1500
1000
500
0
Naked DNA
pAA 60 kDa
p-L-Glu 2-15 kDa
p-L-Glu 50-100 kDa
Naked DNA
pAA 60 kDa
p-L-Glu 2-15 kDa
p-L-Glu 50-100 kDa
Naked DNA
0 0.6 3.0 6.0 12 2.0 6.0 12 6.0
30 µg DNA
0 0.6 3.0 6.0 12 2.0 6.0 12 6.0
100 µg DNA
0
300 ng DNA
Anionic polymer concentration in mg/ml

Fig. 4

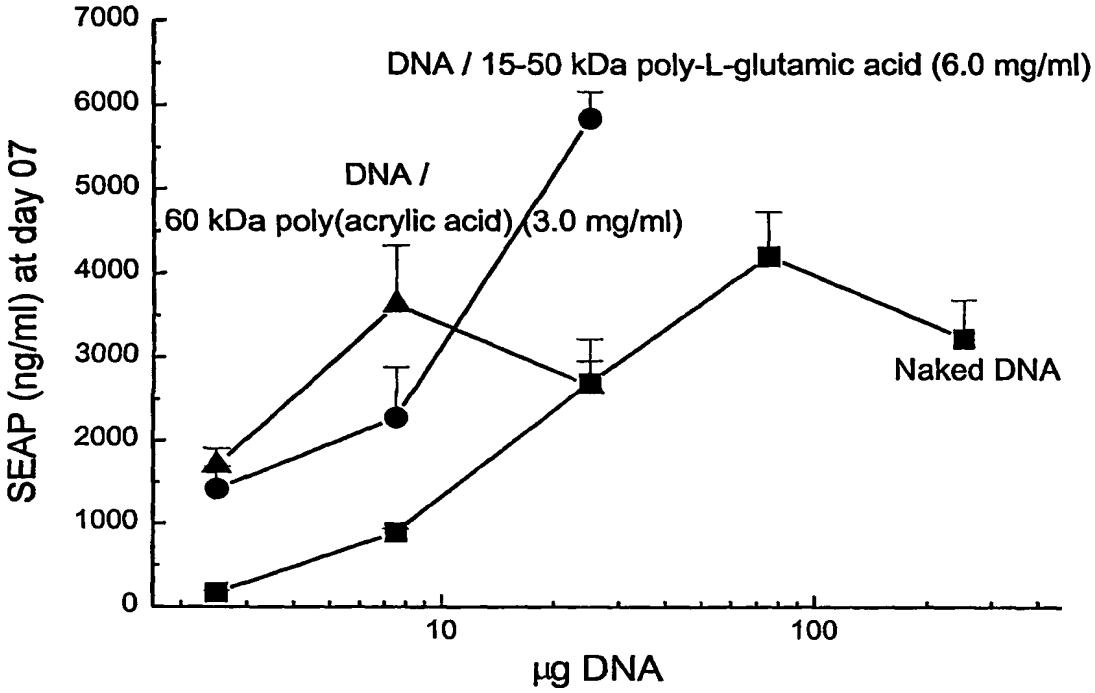

7000
6000
5000
4000
3000
2000
1000
0
SEAP (ng/ml) at day 07
DNA / 15-50 kDa poly-L-glutamic acid (6.0 mg/ml)
DNA /
60 kDa poly(acrylic acid) (3.0 mg/ml)
Naked DNA
10
100
µg DNA

Fig. 5A

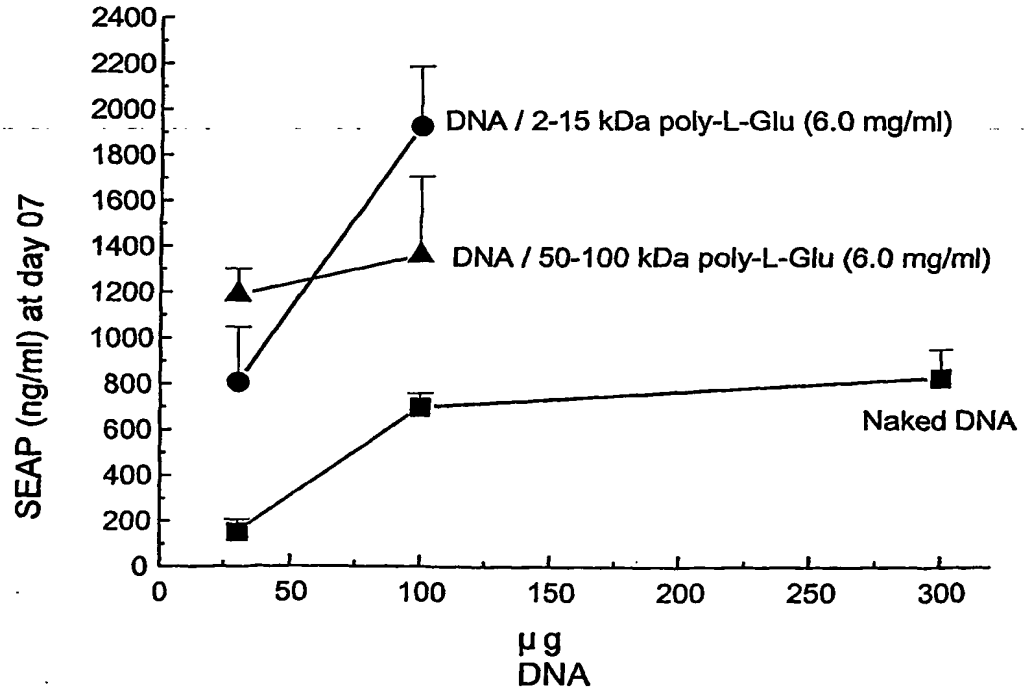

2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
SEAP (ng/ml) at day 07
DNA / 2-15 kDa poly-L-Glu (6.0 mg/ml)
DNA / 50-100 kDa poly-L-Glu (6.0 mg/ml)
Naked DNA
0
50
100
150
200
250
300
µg
DNA

Fig. 5B

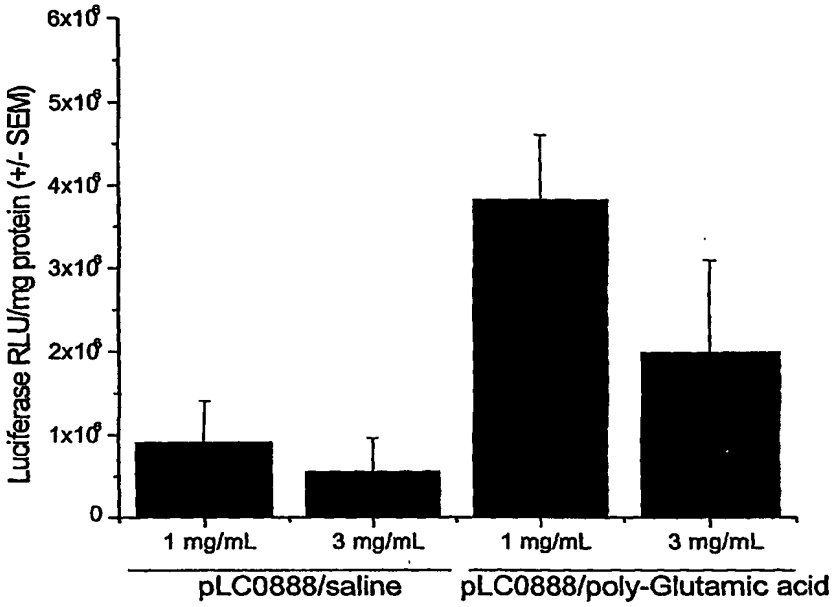

Luciferase RLU/mg protein (+/- SEM)
6x10⁶
5x10⁶
4x10⁶
3x10⁶
2x10⁶
1x10⁶
0
1 mg/mL
3 mg/mL
1 mg/mL
3 mg/mL
pLC0888/saline
pLC0888/poly-Glutamic acid

Fig. 6

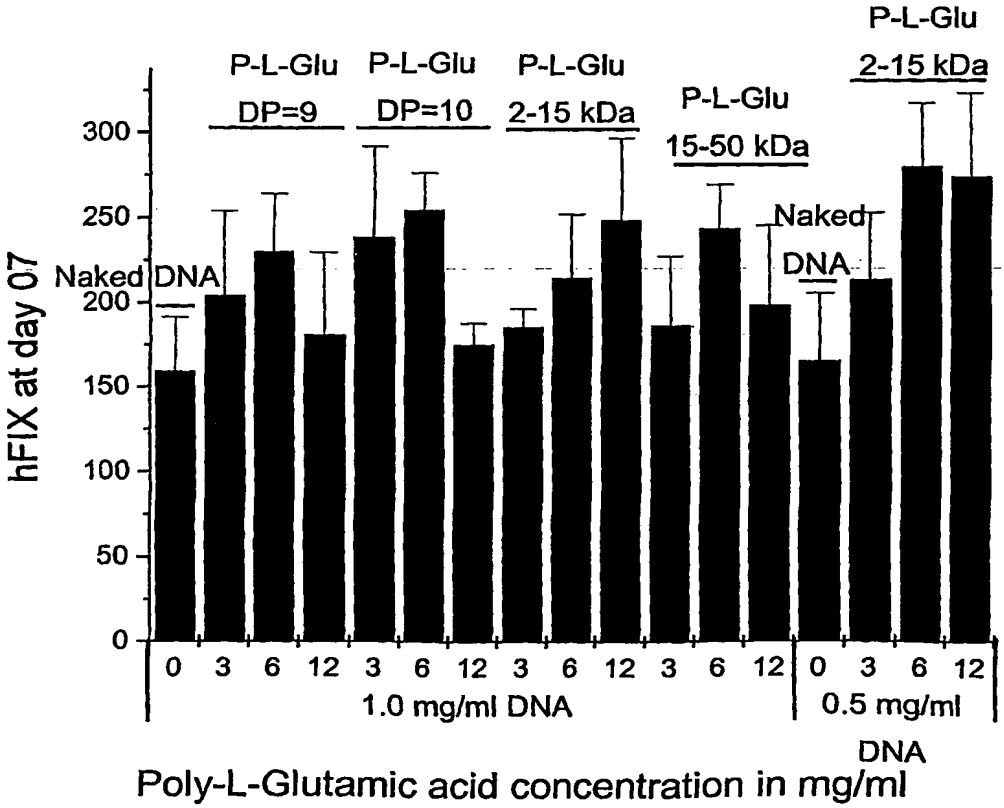

hFIX at day 07
300
250
200
150
100
50
0
Naked DNA
P-L-Glu DP=9
P-L-Glu DP=10
P-L-Glu 2-15 kDa
P-L-Glu 15-50 kDa
Naked DNA
P-L-Glu 2-15 kDa
0 3 6 12 3 6 12 3 6 12 3 6 12 0 3 6 12
1.0 mg/ml DNA
0.5 mg/ml DNA
Poly-L-Glutamic acid concentration in mg/ml

Fig. 7

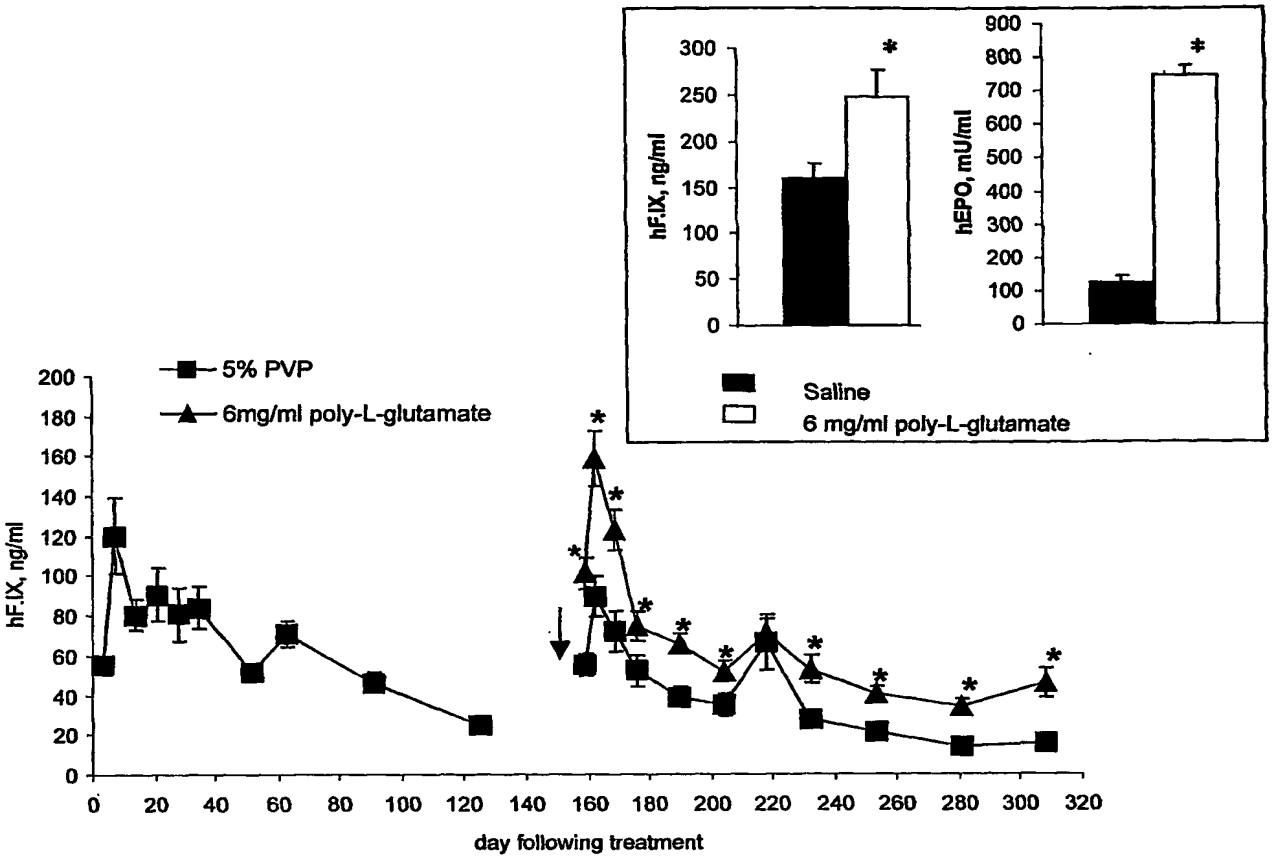

hF.IX, ng/ml
hEPO, mU/ml
Saline
6 mg/ml poly-L-glutamate
5% PVP
6mg/ml poly-L-glutamate
day following treatment

Fig. 8

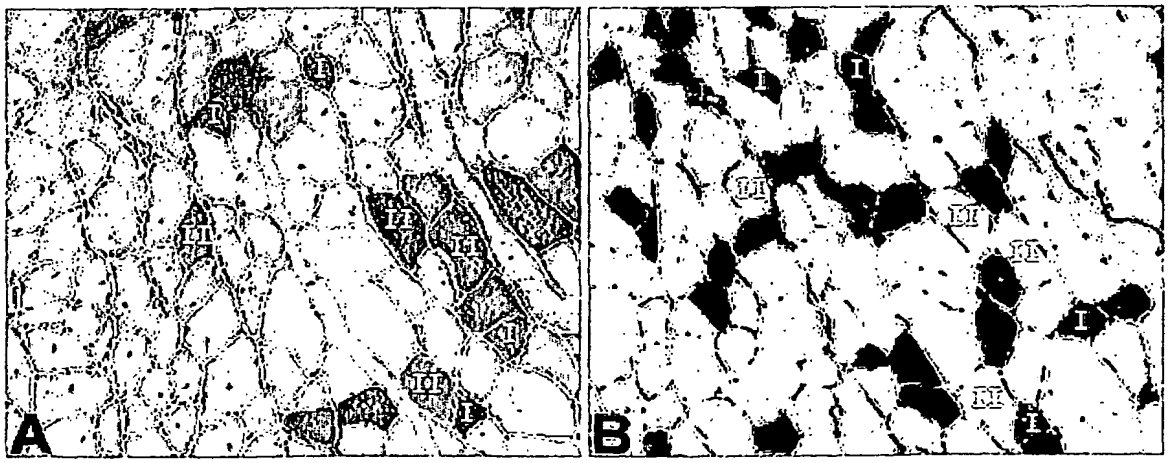

A
B

Fig. 9

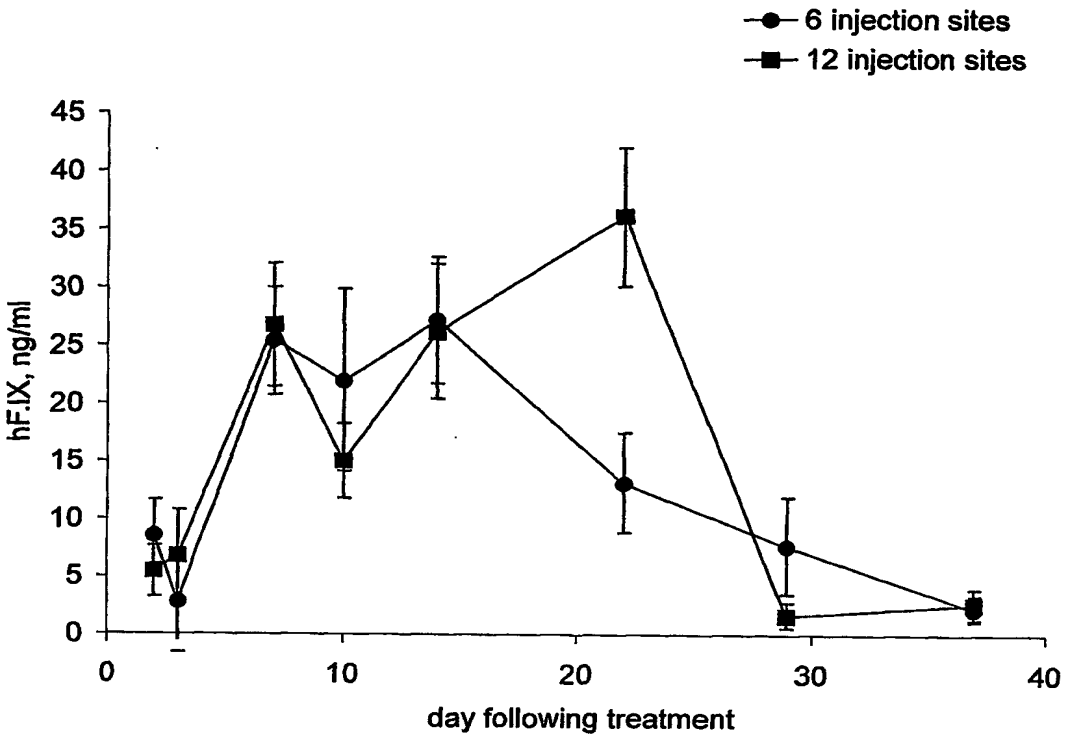


Fig. 10A

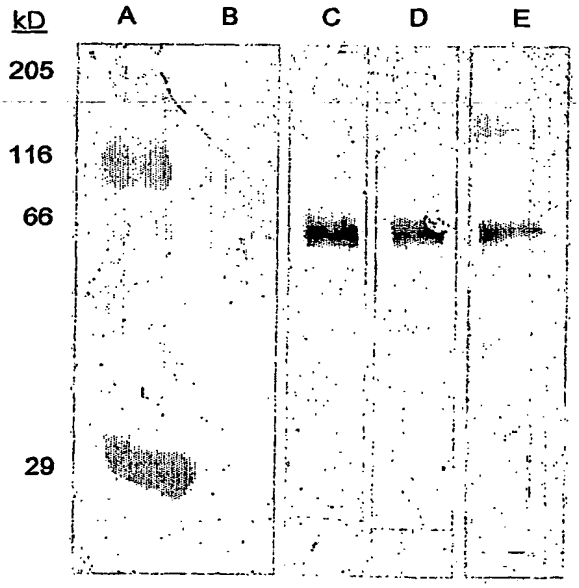


Fig. 10B

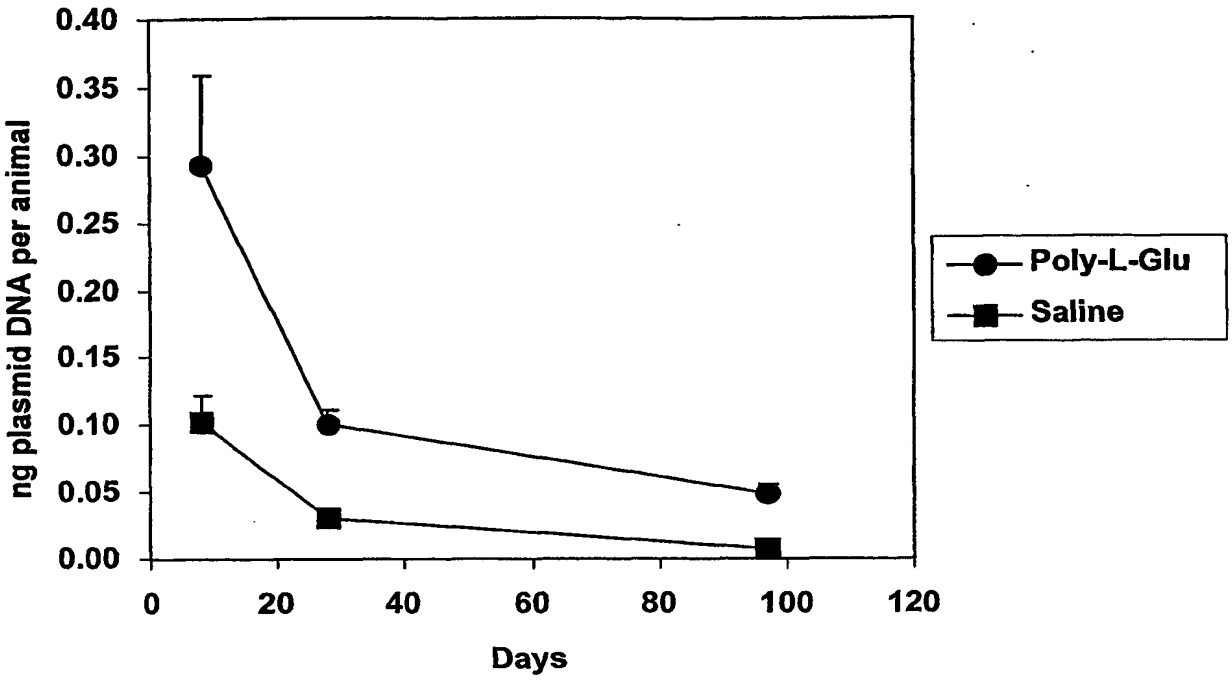

0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
ng plasmid DNA per animal
0
20
40
60
80
100
120
Days
Poly-L-Glu
Saline

Fig. 11

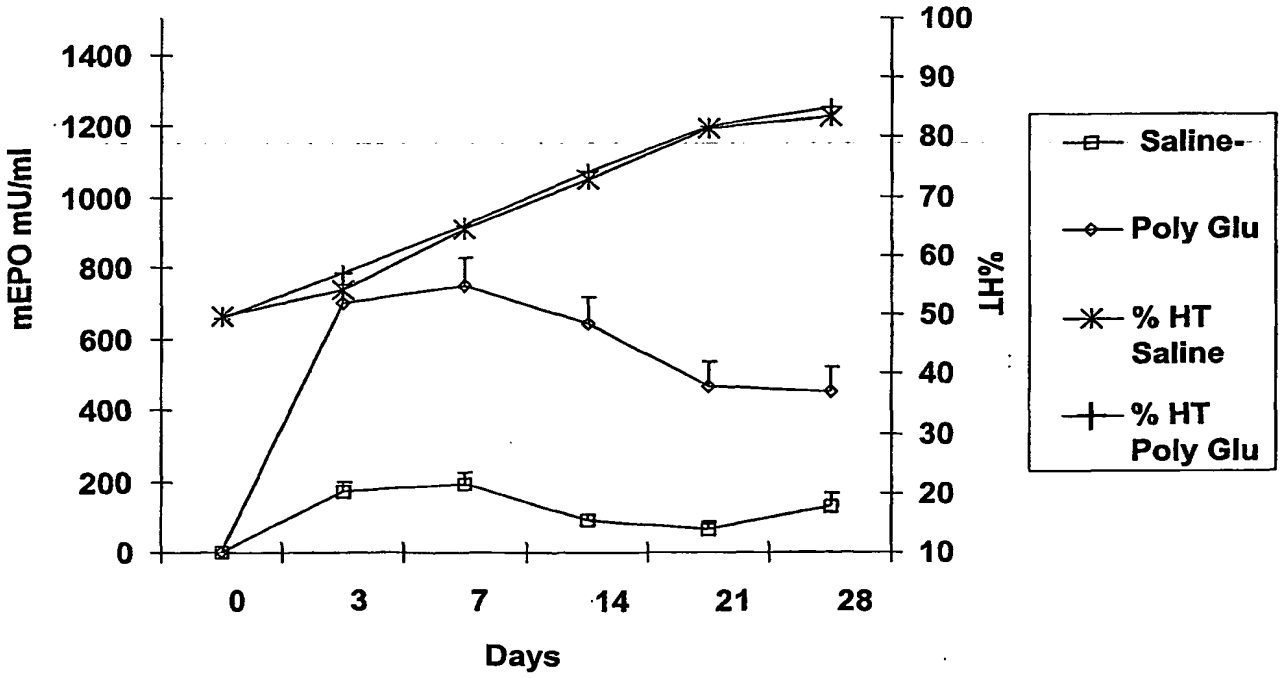

1400
1200
1000
800
600
400
200
0
mEPO mU/ml
100
90
80
70
60
50
40
30
20
10
%HT
0
3
7
14
21
28
Days
Saline-
Poly Glu
% HT Saline
% HT Poly Glu

Fig. 12

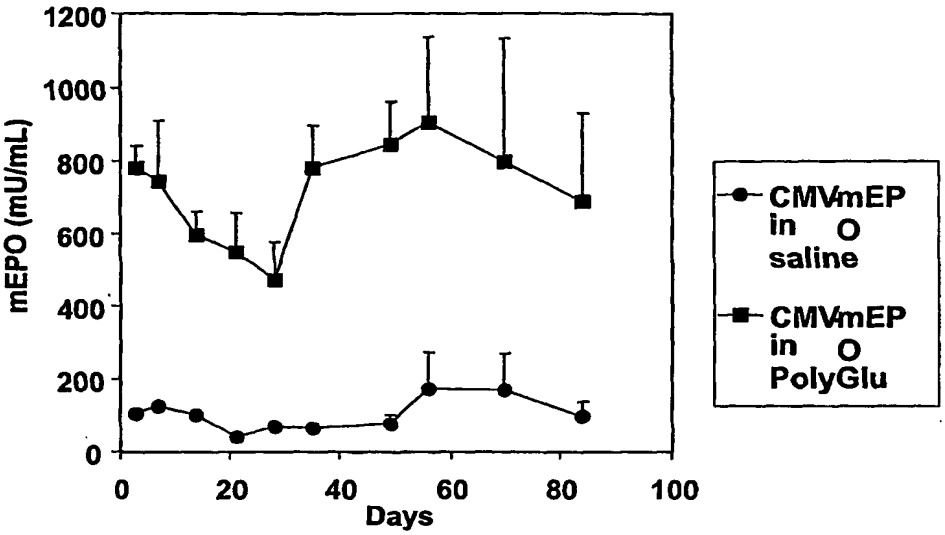


Fig. 13

50 µg dose

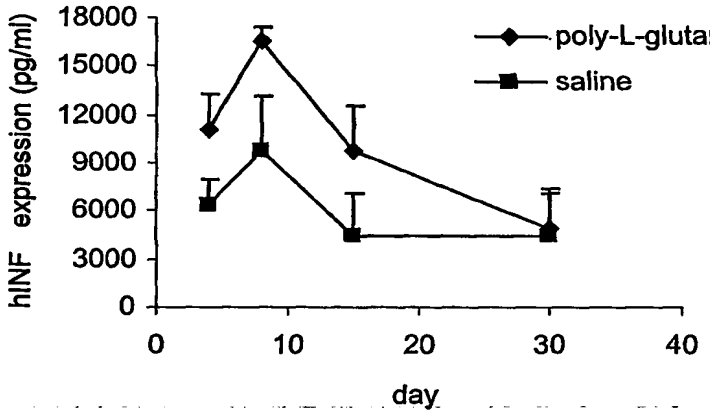


Fig. 14A

5 µg dose

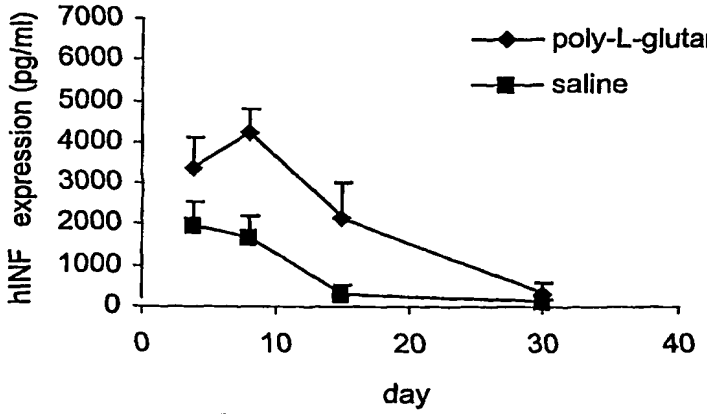


Fig. 14B

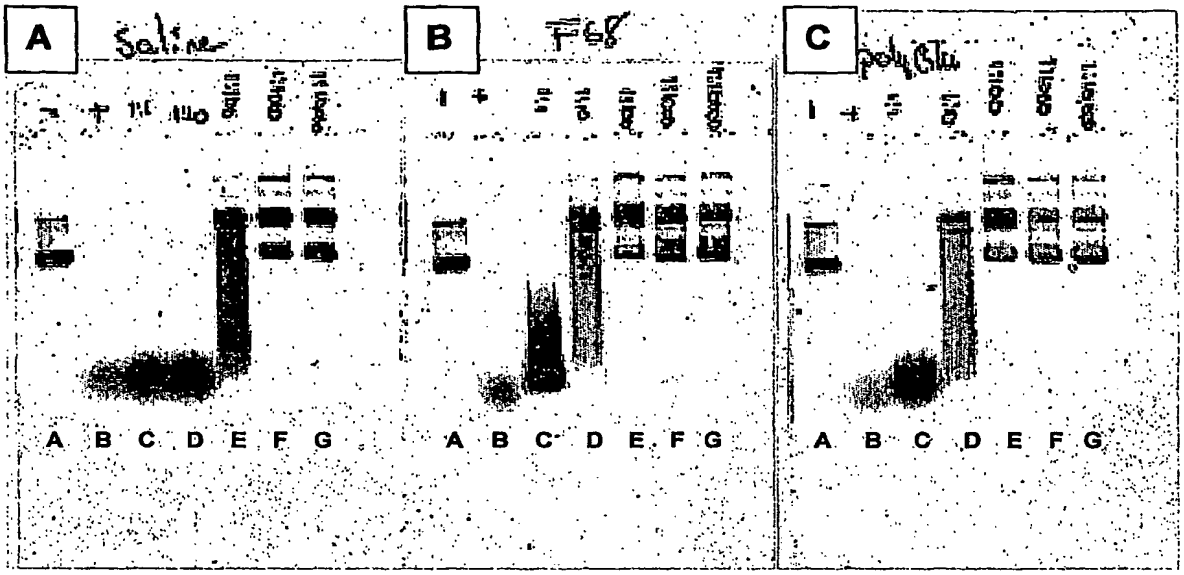

A
Saline
B
F68
C
A B C D E F G
A B C D E F G
A B C D E F G

Fig. 15

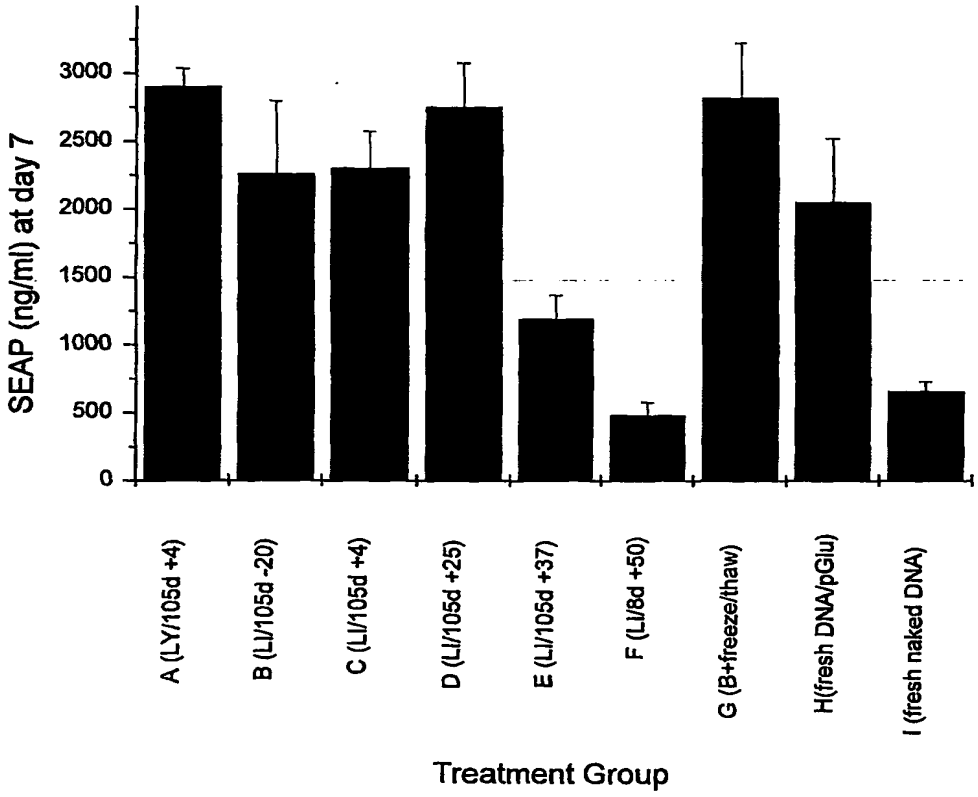

SEAP (ng/ml) at day 7
3000
2500
2000
1500
1000
500
0
A (LY/105d +4)
B (LI/105d -20)
C (LI/105d +4)
D (LI/105d +25)
E (LI/105d +37)
F (LI/8d +50)
G (B+freeze/thaw)
H(fresh DNA/pGlu)
I (fresh naked DNA)
Treatment Group

Fig. 16

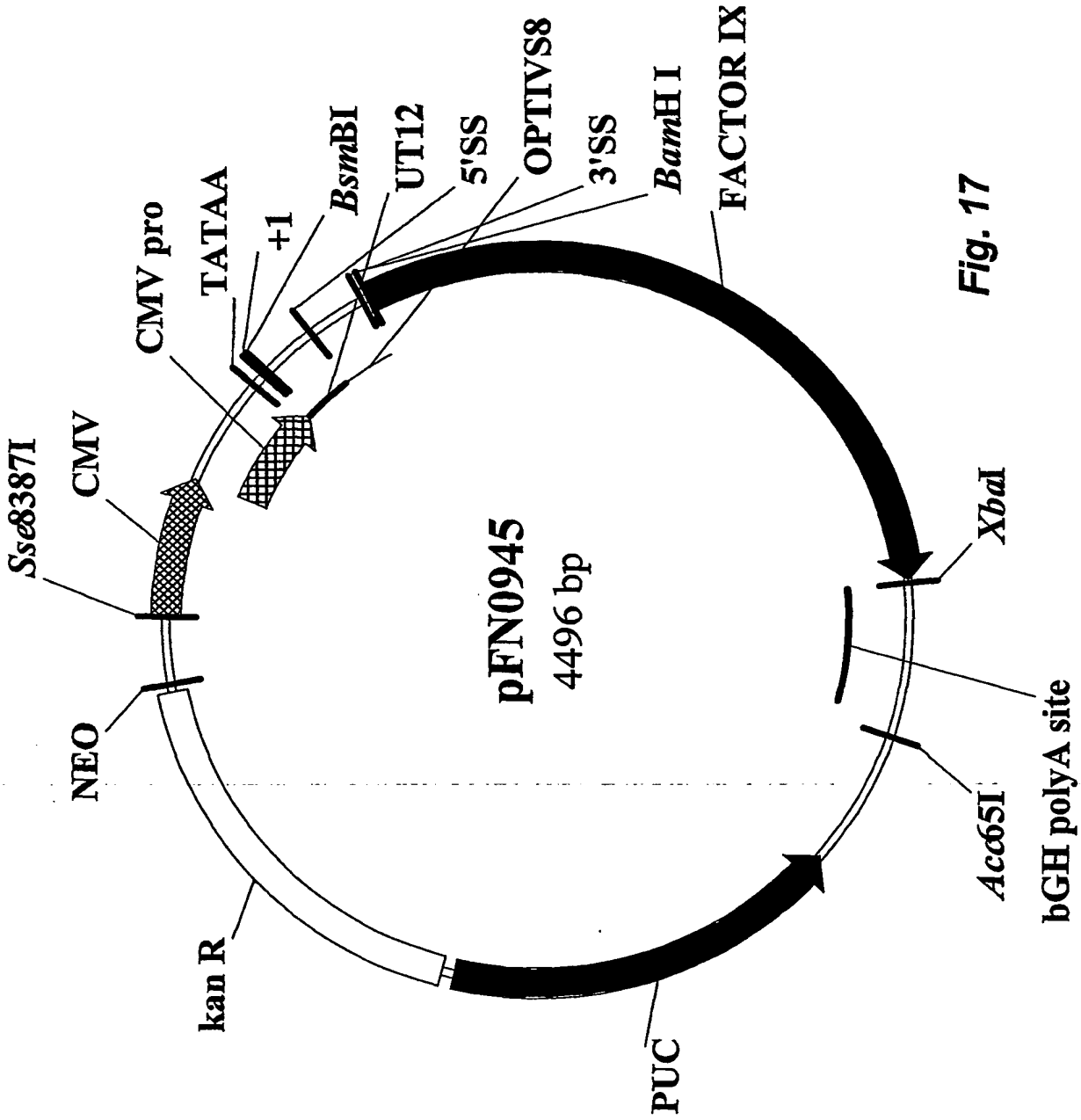

pFN0945
4496 bp
CMV pro
TATAA
+1
BsmBI
UT12
5'SS
OPTIVS8
3'SS
BamH I
FACTOR IX
XbaI
bGH polyA site
Acc65I
PUC
kan R
NEO
CMV
Sse8387I

Fig. 17

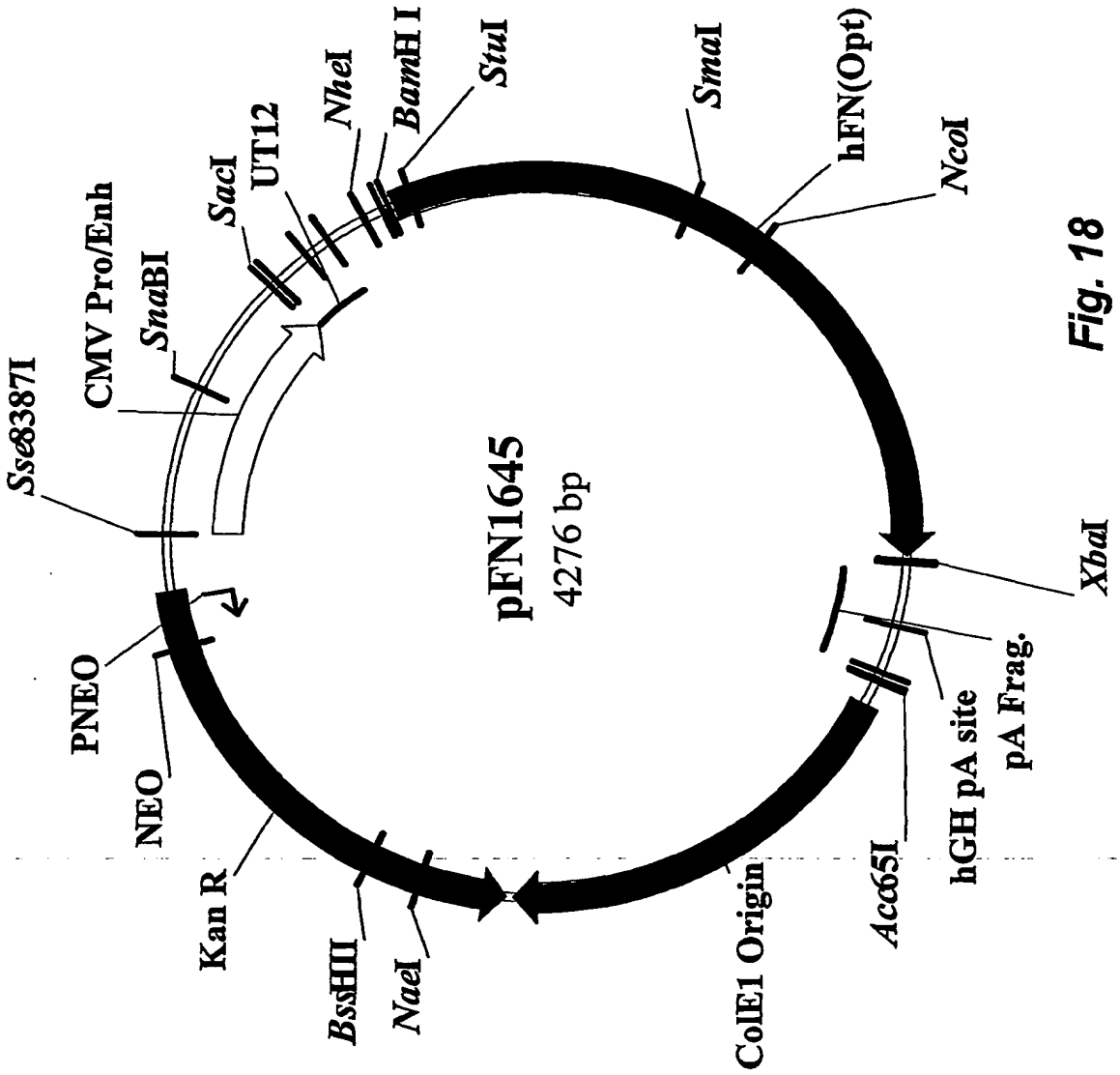
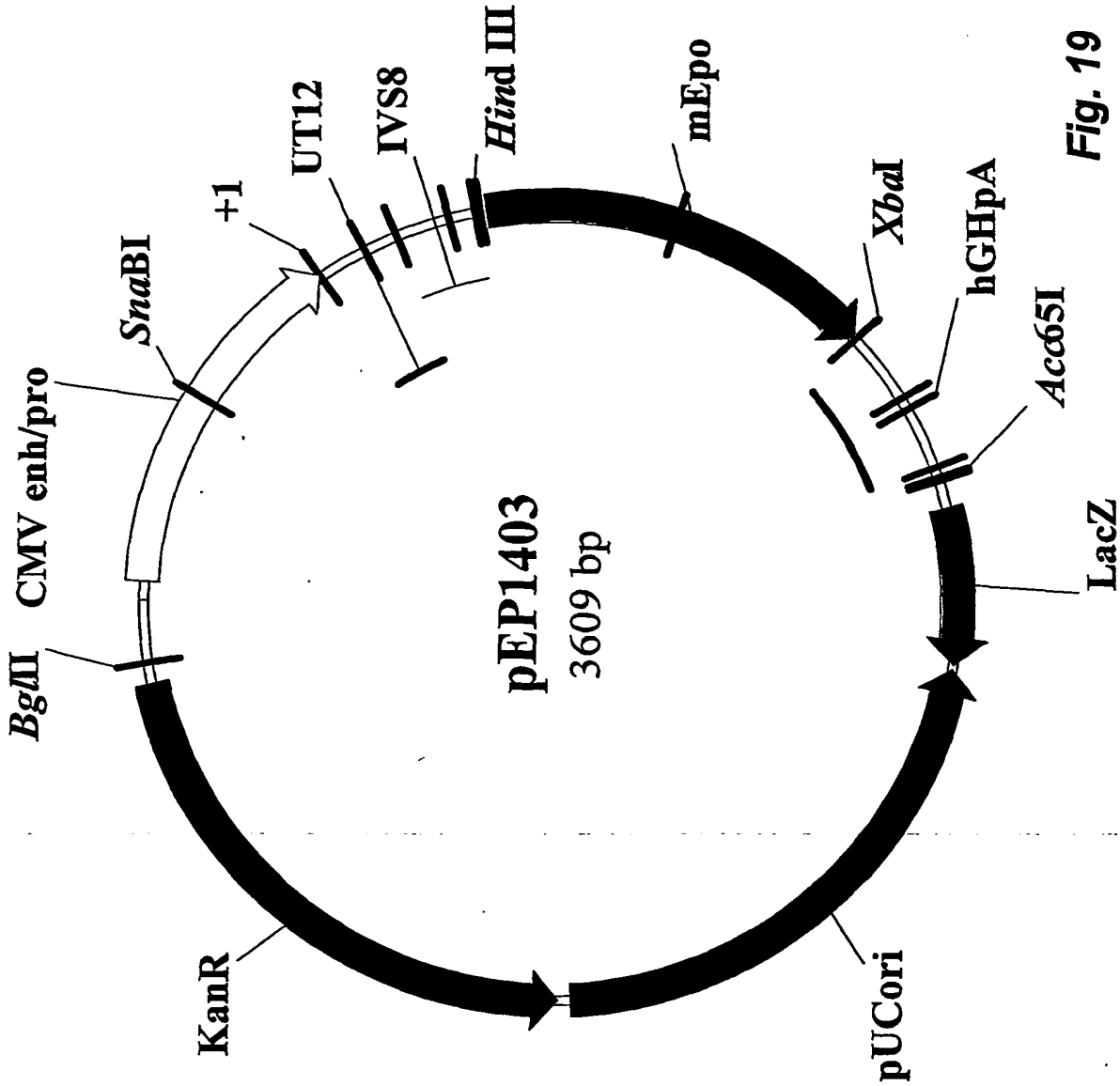

pFN1645
4276 bp
Sse8387I
CMV Pro/Enh
SnaBI
SacI
UT12
NheI
BamH I
StuI
SmaI
hFN(Opt)
NcoI
XbaI
hGH pA site
pA Frag.
Acc65I
ColE1 Origin
NaeI
BssHII
Kan R
NEO
PNEO

Fig. 18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+1
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KanR
BglII

Fig. 19

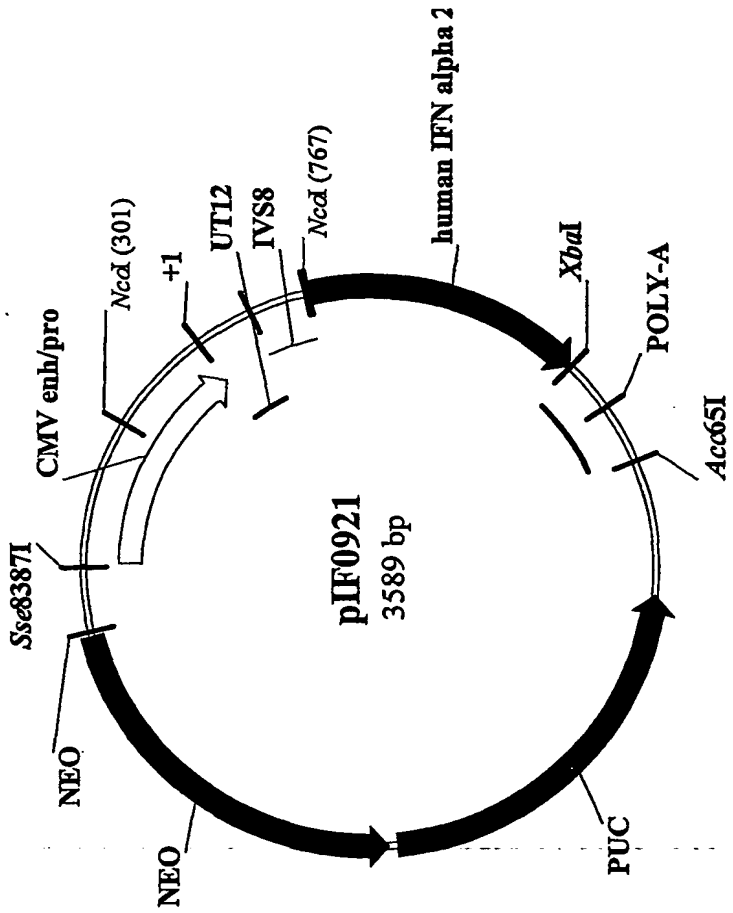
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Fig. 20

SEQUENCE LISTING

<110> Valentis, Inc.

<120> NUCLEIC ACID FORMULATIONS FOR GENE DELIVERY AND METHODS OF USE

<130> 261/125

<140> NOT YET ASSIGNED

<141> HEREWITH

<150> US 60/187,236 and US 60/261,751

<151> 2000-03-03 and 2001-01-16

<160> 4

<170> Microsoft Word

<210> 1

<211> 3589

<212> DNA

<213> Artificial Sequence

<220> CDS

<222> (768) ... (1334)

<223> Expression plasmid pIF0921 encoding for human interferon alpha (768) ... (1334).

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421 ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg gtaggcgtgt
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3421 cgtgcaatcc atcttgttca atcatgcgaa acgatcctca tcctgtctct tgatcagatc
3481 ttgatcccct gcgccatcag atccttggcg gcaagaaagc catccagttt actttgcagg
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<213> Artificial Sequence
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<223> Expression plasmid pEP1403 encoding for mouse erythropoietin (801) ... (1379)
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361 gtggatagcg gtttgactca cggggatttc caagtctcca ccccattgac gtcaatggga
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721 tcagaaactg cagtatctgt atttttgcta gcagtaatac taacggttct ttttttctct
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<222> (782) ... (2167)

<223> Expression plasmid pFN0945 having natural sequence encoding human coagulation factor IX

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<212> DNA

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<223> Expression plasmid pFN1645 having codon optimized sequence encoding for human coagulation factor IX (786) … (2171).

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